EXHIBIT AS

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 766 010 B1

(12) EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention of the grant of the patent:
16.02.2011 Bulletin 2011/07

(21) Application number: **05754344.9**

(22) Date of filing: **28.06.2005**

(51) Int Cl.:
C12N 15/113 (2010.01) ***A61K 48/00*** (2006.01)
A61K 31/7105 (2006.01) ***A61K 31/711*** (2006.01)
C07H 21/00 (2006.01) *A61P 21/00* (2006.01)

(86) International application number:
PCT/AU2005/000943

(87) International publication number:
WO 2006/000057 (05.01.2006 Gazette 2006/01)

(54) **ANTISENSE OLIGONUCLEOTIDES FOR INDUCING EXON SKIPPING AND METHODS OF USE THEREOF**

ANTISENSE-OLIGONUKLEOTIDE ZUR INDUKTION VON EXON-SKIPPING SOWIE VERFAHREN ZUR VERWENDUNG DAVON

OLIGONUCLÉOTIDES ANTISENS PERMETTANT D'INDUIRE UN SAUT D'EXON ET LEURS PROCÉDÉS D'UTILISATION

(84) Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IS IT LI LT LU MC NL PL PT RO SE SI SK TR
Designated Extension States:
HR LV

(30) Priority: **28.06.2004 AU 2004903474**

(43) Date of publication of application:
28.03.2007 Bulletin 2007/13

(60) Divisional application:
10004274.6 / 2 206 781

(73) Proprietor: **THE UNIVERSITY OF WESTERN AUSTRALIA**
Crawley, Western Australia 6009 (AU)

(72) Inventors:
- **WILTON, Stephen, Donald**
  Applecross, Western Australia 6153 (AU)
- **FLETCHER, Sue**
  Bayswater, Western Australia 6053 (AU)
- **MCCLOREY, Graham**
  Bayswater, Western Australia 6053 (AU)

(74) Representative: **Brasnett, Adrian Hugh et al**
Mewburn Ellis LLP
33 Gutter Lane
London
EC2V 8AS (GB)

(56) References cited:
WO-A1-02/24906 **WO-A1-2004/083432**
WO-A2-2004/083446 **AU-A1- 2003 284 638**
AU-B2- 780 517 **CA-A1- 2 507 125**

- **MATSUO M: "DUCHENNE AND BECKER MUSCULAR DYSTROPHY: FROM GENE DIAGNOSIS TO MOLECULAR THERAPY" IUBMB LIFE, vol. 53, no. 3, 1 March 2002 (2002-03-01), pages 147-152, XP009021242 ISSN: 1521-6543**
- **ERRINGTON STEPHEN J ET AL: "Target selection for antisense oligonucleotide induced exon skipping in the dystrophin gene." THE JOURNAL OF GENE MEDICINE, vol. 5, no. 6, June 2003 (2003-06), pages 518-527, XP002559309 ISSN: 1099-498X**
- **AARTSMA-RUS A ET AL: 'Antisense-induced multiexon skipping for Duchenne muscular dystrophy makes more sense' AMERICAN JOURNAL OF HUMAN GENETICS vol. 74, no. 1, 2004, pages 83 - 92, XP008084158**
- **DE ANGELIS F G ET AL: 'Chimeric snRNA molecules carrying antisense sequences against the splice junctions of exon 51 of the dystrophin pre-mRNA induce exon skipping and restoration of a dystrophin synthesis in Delta 48-50 DMD cells.' PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES vol. 99, no. 14, 2002, pages 9456 - 9461, XP008115971**

- **AARTSMA-RUS A ET AL: 'Targeted exon skipping as a potential gene correction therapy for Duchenne muscular dystrophy.' NEUROMUSCULAR DISORDERS vol. 12, 2002, pages 71 - 77, XP008116183**
- **AARTSMA-RUS A ET AL: 'Therapeutic antisense-induced exon skipping in cultured muscle cells from six different DMD patients.' HUMAN MOLECULAR GENETICS vol. 12, no. 8, 2003, pages 907 - 914, XP008084159**

Remarks:

The file contains technical information submitted after the application was filed and not included in this specification

Description

Field of the Invention

[0001] The present invention relates to novel antisense compounds and compositions suitable for facilitating exon skipping. It also provides methods for inducing exon skipping using the novel antisense compounds as well as therapeutic compositions adapted for use in the methods of the invention..

Background Art

[0002] Significant effort is currently being expended researching methods for suppressing or compensating for disease-causing mutations in genes. Antisense technologies are being developed using a range of chemistries to affect gene expression at a variety of different levels (transcription, splicing, stability, translation). Much of that research has focused on the use of antisense compounds to correct or compensate for abnormal or disease-associated genes in a myriad of different conditions.

[0003] Antisense molecules are able to inhibit gene expression with exquisite specificity and because of this many research efforts concerning oligonucleotides as modulators of gene expression have focused on inhibiting the expression of targeted genes such as oncogenes or viral genes. The antisense oligonucleotides are directed either against RNA (sense strand) or against DNA where they form triplex structures inhibiting transcription by RNA polymerase II. To achieve a desired effect in specific gene down-regulation, the oligonucleotides must either promote the decay of the targeted mRNA or block translation of that mRNA, thereby effectively preventing *de novo* synthesis of the undesirable target protein.

[0004] Such techniques are not useful where the object is to up-regulate production of the native protein or compensate for mutations which induce premature termination of translation such as nonsense or frame-shifting mutations.

[0005] Furthermore, in cases where a normally functional protein is prematurely terminated because of mutations therein, a means for restoring some functional protein production through antisense technology has been shown to be possible through intervention during the splicing processes (Sierakowska H, et al., (1996) Proc Natl Acad Sci USA 93,12840-12844; Wilton SD, et al., (1999) Neuromusc Disorders 9, 330-338; van Deutekom JC et al., (2001) Human Mol Genet 10, 1547-1554). In these cases, the defective gene transcript should not be subjected to targeted degradation so the antisense oligonucleotide chemistry should not promote target mRNA decay.

[0006] In a variety of genetic diseases, the effects of mutations on the eventual expression of a gene can be modulated through a process of targeted exon skipping during the splicing process. The splicing process is directed by complex multi-particle machinery that brings adjacent exon-intron junctions in pre-mRNA into close proximity and performs cleavage of phosphodiester bonds at the ends of the introns with their subsequent reformation between exons that are to be spliced together. This complex and highly precise process is mediated by sequence motifs in the pre-mRNA that are relatively short semi-conserved RNA segments to which bind the various nuclear splicing factors that are then involved in the splicing reactions. By changing the way the splicing machinery reads or recognises the motifs involved in pre-mRNA processing, it is possible to create differentially spliced mRNA molecules. It has now been recognised that the majority of human genes are alternatively spliced during normal gene expression, although the mechanisms invoked have not been identified. Using antisense oligonucleotides, it has been shown that errors and deficiencies in a coded mRNA could be bypassed or removed from the mature gene transcripts.

[0007] In nature, the extent of genetic deletion or exon skipping in the splicing process is not fully understood, although many instances have been documented to occur, generally at very low levels (Sherrat TG, et al., (1993) Am J Hum Genet 53, 1007-1015). However, it is recognised that if exons associated with disease-causing mutations can be specifically deleted from some genes, a shortened protein product can sometimes be produced that has similar biological properties of the native protein or has sufficient biological activity to ameliorate the disease caused by mutations associated with the target exon (Lu QL, et al., (2003) Nature Medicine 9, 1009-1014; Aartsma-Rus A et al., (2004) Am J Hum Genet 74: 83-92).

[0008] This process of targeted exon skipping is likely to be particularly useful in long genes where there are many exons and introns, where there is redundancy in the genetic constitution of the exons or where a protein is able to function without one or more particular exons (e.g. with the dystrophin gene, which consists of 79 exons; or possibly some collagen genes which encode for repeated blocks of sequence or the huge nebulin or titin genes which are comprised of -80 and over 370 exons, respectively).

[0009] Efforts to redirect gene processing for the treatment of genetic diseases associated with truncations caused by mutations in various genes have focused on the use of antisense oligonucleotides that either: (1) fully or partially overlap with the elements involved in the splicing process; or (2) bind to the pre-mRNA at a position sufficiently close to the element to disrupt the binding and function of the splicing factors that would normally mediate a particular splicing reaction which occurs at that element (e.g., binds to the pre-mRNA at a position within 3, 6, or 9 nucleotides of the

element to be blocked).

[0010] For example, modulation of mutant dystrophin pre-mRNA. splicing with antisense oligoribonucleotides has been reported both *in vitro* and *in vivo.* In one type of dystrophin mutation reported in Japan, a 52-base pair deletion mutation causes exon 19 to be removed with the flanking introns during the splicing process (Matsuo et al., (1991) J Clin Invest. 87:2127-2131). An *in vitro* minigene splicing system has been used to show that a 31-mer 2'-O-methyl oligoribonucleotide complementary to the 5' half of the deleted sequence in dystrophin Kobe exon 19 inhibited splicing of wild-type *pre-mRNA* (Takeshima et al. (1995), J. Clin. Invest., 95, 515-520). The same oligonucleotide was used to induce exon skipping from the native dystrophin gene transcript in human cultured lymphoblastoid cells.

[0011] Dunckley et al., (1997) Nucleosides & Nucleotides, 16, 1665-1668 described *in vitro* constructs for analysis of splicing around exon 23 of mutated dystrophin in the *mdx* mouse mutant, a model for muscular dystrophy. Plans to analyse these constructs *in vitro* using 2' modified oligonucleotides targeted to splice sites within and adjacent to mouse dystrophin exon 23 were discussed, though no target sites or sequences were given.

[0012] 2'-O-methyl oligoribonucleotides were subsequently reported to correct dystrophin deficiency in myoblasts from the *mdx* mouse from this group. An antisense oligonucleotide targeted to the 3' splice site of murine dystrophin intron 22 was reported to cause skipping of the mutant exon as well as several flanking exons and created a novel in-frame dystrophin transcript with a novel internal deletion. This mutated dystrophin was expressed in 1-2% of antisense treated mdx myotubes. Use of other oligonucleotide modifications such as 2'-O-methoxyethyl phosphodiesters are described (Dunckley et al. (1998) Human Mol. Genetics, 5, 1083-90).

[0013] Thus, antisense molecules may provide a tool in the treatment of genetic disorders such as Duchenne Muscular Dystrophy (DMD). However, attempts to induce exon skipping using antisense molecules have had mixed success. Studies on dystrophin exon 19, where successful skipping of that exon from the dystrophin pre-mRNA was achieved using a variety of antisense molecules directed at the flanking splice sites or motifs within the exon involved in exon definition as described by Errington et al. (2003) J Gen Med 5, 518-527".

[0014] In contrast to the apparent ease of exon 19 skipping, the first report of exon 23 skipping in the *mdx* mouse by Dunckley *et al.,* (1998) is now considered to be reporting only a naturally occurring revertant transcript or artefact rather than any true antisense activity. In addition to not consistently generating transcripts missing exon 23, Dunckley *et al.,* (1998) did not show any time course of induced exon skipping, or even titration of antisense oligonucleotides, to demonstrate dose dependent effects where the levels of exon skipping corresponded with increasing or decreasing amounts of antisense oligonucleotide. Furthermore, this work could not be replicated by other researchers.

[0015] The first example of specific and reproducible exon skipping in the *mdx* mouse model was reported by Wilton et al., (1999) Neuromuscular Disorders 9, 330-338. By directing an antisense molecule to the donor splice site, consistent and efficient exon 23 skipping was induced in the dystrophin mRNA within 6 hours of treatment of the cultured cells. Wilton et al., (1999), also describe targeting the acceptor region of the mouse dystrophin pre-mRNA with longer antisense oligonucleotides and being unable to repeat the published results of Dunckley et al., (1998). No exon skipping, either 23 alone or multiple removal of several flanking exons, could be reproducibly detected using a selection of antisense oligonucleotides directed at the acceptor splice site of intron 22.

[0016] While the first antisense oligonucleotide directed at the intron 23 donor splice site induced consistent exon skipping in primary cultured myoblasts, this compound was found to be much less efficient in immortalized cell cultures expressing higher levels of dystrophin. However, with refined targeting and antisense oligonucleotide design, the efficiency of specific exon removal was increased by almost an order of magnitude (see Mann CJ et al., (2002) J Gen Med 4, 644654).

[0017] Other disclosures relating to DMD therapy include CA 2507125 and Matsuo M: "Duchenne And Becker Muscular Dystrophy: From Gene Diagnosis To Molecular Therapy" IUBMB LIFE, vol.53, no. 3, 1 March 2002 (2002-03-01), pages 147-152, as well as WO 2004/083446, published on 30 September 2004.

[0018] Thus, there remains a need to provide antisense oligonucleotides capable of binding to and modifying the splicing of a target nucleotide sequence. Simply directing the antisense oligonucleotides to motifs presumed to be crucial for splicing is no guarantee of the efficacy of that compound in a therapeutic setting.

Summary of the Invention

[0019] The present invention provides an antisense oligonucleotide that binds to the human dystrophin gene to induce exon skipping in the dystrophin gene, consisting of the sequence of SEQ ID NO: 181, optionally wherein the uracil bases (U) are thymine bases (T).

[0020] The invention further provides a composition comprising an antisense molecule according to the invention one or more pharmaceutically acceptable carriers and/or diluents.

[0021] The invention further provides an antisense molecule or composition according to the invention for use in a method of treatment of muscular dystrophy in a patient.

[0022] The invention is further defined in the accompanying claims.

[0023] The choice of target selection plays a crucial role in the efficiency of exon skipping and hence its subsequent application of a potential therapy. Simply designing antisense molecules to target regions of pre-mRNA presumed to be involved in splicing is no guarantee of inducing efficient and specific exon skipping. The most obvious or readily defined targets for splicing intervention are the donor and acceptor splice sites although there are less defined or conserved motifs including exonic splicing enhancers, silencing elements and branch points.

[0024] The acceptor and donor splice sites have consensus sequences of about 16 and 8 bases respectively (see Figure 1 for schematic representation of motifs and domains involved in exon recognition, intron removal and the splicing process).

[0025] The invention also addresses the use of purified and isolated antisense oligonucleotides of the invention, for the manufacture of a medicament for treatment of a genetic disease.

[0026] The invention may be used for treating a condition characterised by Duchenne muscular dystrophy, by administering to a patient in need of treatment an effective amount of an appropriately designed antisense oligonucleotide of the invention, relevant to the particular genetic lesion in that patient.

[0027] Further, the invention may be used for prophylactically treating a patient to prevent or at least minimise Duchene muscular dystrophy, comprising the step of: administering to the patient an effective amount of an antisense oligonucleotide or a pharmaceutical composition comprising one or more of these biological molecules.

[0028] Also described herein are kits for treating a genetic disease, which kits comprise at least a antisense oligonucleotide of the present invention, packaged in a suitable container and instructions for its use.

[0029] Other aspects and advantages of the invention will become apparent to those skilled in the art from a review of the ensuing description, which proceeds with reference to the following figures.

Brief Description of the Drawings

[0030]

Figure 1 Schematic representation of motifs and domains involved in exon recognition, intron removal and the splicing process.

Figure 2. Diagrammatic representation of the concept of antisense oligonucleotide induced exon skipping to bypass disease-causing mutations (not drawn to scale). The hatched box represents an exon carrying a mutation that prevents the translation of the rest of the mRNA into a protein. The solid black bar represents an antisense oligonucleotide that prevents inclusion of that exon in the mature mRNA.

[0031] The 2'-O-methyl phosphorothioate antisense oligonucleotides that have been used to study induced exon skipping during the processing of the dystrophin pre-mRNA are described in Table 1 of the examples. Since these 2'-O-methyl antisense oligonucleotides are more RNA-like, U represents uracil. With other antisense chemistries such as peptide nucleic acids or morpholinos, these U bases may be shown as "T".

Detailed Description of the Invention

General

[0032] Those skilled In the art will appreciate that the Invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variation and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in the specification, individually or collectively and any and all combinations or any two or more of the steps or features.

[0033] The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purpose of exemplification only. Functionally equivalent products, compositions and methods are clearly within the scope of the invention as described herein.

[0034] Sequence identity numbers (SEQ ID NO:) containing nucleotide and amino acid sequence information included in this specification are collected at the end of the description and have been prepared using the programme Patentin Version 3.0. Each nucleotide or amino acid sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier (e.g. <210>1, <210>2, etc.). The length, type of sequence and source organism for each nucleotide or amino acid sequence are indicated by information provided in the numeric indicator fields <211>, <212> and <213>, respectively. Nucleotide and amino acid sequences referred to in the specification are defined by the information provided In numeric indicator field <400> followed by the sequence identifier (e.g. <400>1, <400>2, etc.).

[0035] An antisense molecules nomenclature system was proposed and published to distinguish between the different

antisense molecules (see Mann et al., (2002) J Gen Med 4, 644-654). This nomenclature became especially relevant when testing several slightly different antisense molecules, all directed at the same target region, as shown below:

H # A/D (x : y).

[0036] The first letter designates the species (e.g. H: human, M: murine, C: canine)
"#" designates target dystrophin exon number.

[0037] "A/D" indicates acceptor or donor splice site at the beginning and end of the exon, respectively.

[0038] (x y) represents the annealing coordinates where "-" or "+" indicate intronic or exonic sequences respectively. As an example, A(-6+18) would indicate the last 6 bases of the intron preceding the target exon and the first 18 bases of the target exon. The closest splice site would be the acceptor so these coordinates would be preceded with an "A". Describing annealing coordinates at the donor splice site could be D(+2-18) where the last 2 exonic bases and the first 18 intronic bases correspond to the annealing site of the antisense molecule. Entirely exonic annealing coordinates that would be represented by A(+65+85), that is the site between the 65^{th} and 85^{th} nucleotide from the start of that exon.

[0039] No admission is made that any of the references constitute prior art or are part of the common general knowledge of those working In the field to which this invention relates.

[0040] As used necessarily herein the term "derived" and "derived from" shall be taken to Indicate that a specific integer may be obtained from a particular source *albeit* not directly from that source.

[0041] Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

[0042] Other definitions for selected terms used herein may be found within the detailed description of the invention and apply throughout Unless otherwise defined, all other scientific and technical terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which the invention belongs.

Description of the Preferred Embodiment

[0043] When antisense molecule(s) are targeted to nucleotide sequences involved in splicing in exons within pre-mRNA sequences, normal splicing of the exon may be inhibited causing the splicing machinery to by-pass the entire mutated exon from the mature mRNA. The concept of antisense oligonucleotide induced exon skipping Is shown in Figure 2. In many genes, deletion of an entire exon would lead to the production of a non-functional protein through the loss of important functional domains or the disruption of the reading frame. In some proteins, however, it is possible to shorten the protein by deleting one or more exons, without disrupting the reading frame, from within the protein without seriously altering the biological activity of the protein. Typically, such proteins have a structural role and or possess functional domains at their ends. The present invention describes antisense molecules capable of binding to specified dystrophin pre-mRNA targets and re-directing processing of that gene.

Antisense Molecules

[0044] According to a first aspect of the invention, there is provided antisense molecules as defined in the claims capable of binding to a selected target to Induce exon skipping. Designing antisense molecules to completely mask consensus splice sites may not necessarily generate any skipping of the targeted exon. Furthermore, the inventors have discovered that size or length of the antisense oligonucleotide itself is not always a primary factor when designing antisense molecules. With some targets such as exon 19, antisense oligonucleotides as short as 12 bases were able to induce exon skipping, albeit not as efficiently as longer (20-31 bases) oligonucleotides. In some other targets, such as murine dystrophin exon 23, antisense oligonucleotides only 17 residues long were able to induce more efficient skipping than another overlapping compound of 25 nucleotides.

[0045] The inventors have also discovered that there does not appear to be any standard motif that can be blocked or masked by antisense molecules to redirect splicing. In some exons, such as mouse dystrophin exon 23, the donor splice site was the most amenable to target to re-direct skipping of that exon. It should be noted that designing and testing a series of exon 23 specific antisense molecules to anneal to overlapping regions of the donor splice site showed considerable variation in the efficacy of induced exon skipping. As reported In Mann et al., (2002) there was a significant variation in the efficiency of bypassing the nonsense mutation depending upon antisense oligonucleotide annealing ("improved antisense oligonucleotide induced exon skipping in the mdx mouse model of muscular dystrophy". J Gen Med 4: 644-654). Targeting the acceptor site of exon 23 or several internal domains was not found to Induce any consistent exon 23 skipping.

[0046] In other exons targeted for removal, masking the donor splice site did not induce any exon skipping. However, by directing antisense molecules to the acceptor splice site (human exon 8) strong and sustained exon skipping was induced. It should be noted that removal of human exon 8 was tightly linked with the co-removal of exon 9. There is no strong sequence homology between the exon 8 antisense oligonucleotides and corresponding regions of exon 9 so it does not appear to be a matter of cross reaction. Rather the splicing of these two exons is inextricably linked. This is not an isolated instance as the same effect is observed in canine cells where targeting exon 8 for removal also resulted in the skipping of exon 9. Targeting exon 23 for removal in the mouse dystrophin pre-mRNA also results in the frequent removal of exon 22 as well. This effect occurs in a dose dependent manner and also indicates close coordinated processing of 2 adjacent exons.

[0047] In other targeted exons, antisense molecules directed at the donor or acceptor splice sites did not induce exon skipping while annealing antisense molecules to intra-exonic regions (i.e. exon splicing enhancers within human dystrophin exon 6) was most efficient at inducing exon skipping. Some exons, both mouse and human exon 19 for example, are readily skipped by targeting antisense molecules to a variety of motifs. That is, targeted exon skipping Is induced after using antisense oligonucleotides to mask donor and acceptor splice sites or exon splicing enhancers.

[0048] To identify and select antisense oligonucleotides suitable for use in the modulation of exon skipping, a nucleic acid sequence whose function is to be modulated must first be identified. This may be, for example, a gene (or mRNA transcribed form the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent Within the context of the present invention, preferred target site(s) are those involved in mRNA splicing (i.e. splice donor sites, splice acceptor sites, or exonic splicing enhancer elements). Splicing branch points and exon recognition sequences or splice enhancers are also potential target sites for modulation of mRNA splicing.

[0049] Preferably, the present invention aims to provide antisense molecules capable of binding to a selected target in the dystrophin pre-mRNA to induce efficient and consistent exon skipping. Duchenne muscular dystrophy arises from mutations that preclude the synthesis of a functional dystrophin gene product. These Duchenne muscular dystrophy gene defects are typically nonsense mutations or genomic rearrangements such as deletions, duplications or micro-deletions or insertions that disrupt the reading frame. As the human dystrophin gene is a large and complex gene with the 79 exons being spliced together to generate a mature mRNA with an open reading frame of approximately 11,000 bases, there are many positions where these mutations can occur. Consequently, a comprehensive antisense oligonucleotide based therapy to address many of the different disease-causing mutations in the dystrophin gene will require that many exons can be targeted for removal during the splicing process.

[0050] Within the context of the present invention, preferred target site(s) are those involved in mRNA splicing (i.e. splice donor sites, splice acceptor sites or exonic splicing enhancer elements). Splicing branch points and exon recognition sequences or splice enhancers are also potential target sites for modulation of mRNA splicing.

[0051] The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridisable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense molecule need not be 100% complementary to that of its target sequence to be specifically hybridisable. An antisense molecule is specifically hybridisable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment, and in the case of *in vitro* assays, under conditions in which the assays are performed.

[0052] While the above method may be used to select antisense molecules capable of deleting any exon from within a protein that is capable of being shortened without affecting its biological function, the exon deletion should not lead to a reading frame shift in the shortened transcribed mRNA. Thus, if in a linear sequence of three exons the end of the first exon encodes two of three nucleotides in a codon and the next exon is deleted then the third exon in the linear sequence must start with a single nucleotide that is capable of completing the nucleotide triplet for a codon. If the third exon does not commence with a single nucleotide there will be a reading frame shift that would lead to the generation of truncated or a non-functional protein.

[0053] It will be appreciated that the codon arrangements at the end of exons in structural proteins may not always break at the end of a codon, consequently there may be a need to delete more than one exon from the pre-mRNA to ensure in-frame reading of the mRNA. In such circumstances, a plurality of antisense oligonucleotides may need to be selected by the method of the invention wherein each is directed to a different region responsible for inducing splicing in the exons that are to be deleted.

[0054] The length of an antisense molecule may vary so long as it is capable of binding selectively to the intended location within the pre-mRNA molecule. The length of such sequences can be determined in accordance with selection procedures described herein. Generally, the antisense molecule will be from about 10 nucleotides in length up to about

50 nucleotides in length. It will be appreciated however that any length of nucleotides within this range may be used in the method. Preferably, the length of the antisense molecule is between 17 to 30 nucleotides in length.

[0055] In order to determine which exons can be connected in a dystrophin gene, reference should be made to an exon boundary map. Connection of one exon with another is based on the exons possessing the same number at the 3' border as is present at the 5' border of the exon to which it is being connected. Therefore, if exon 7 were deleted, exon 6 must connect to either exons 12 or 18 to maintain the reading frame. Thus, antisense oligonucleotides would need to be selected which redirected splicing for exons 7 to 11 in the first instance or exons 7 to 17 in the second instance. Another and somewhat simpler approach to restore the reading frame around an exon 7 deletion would be to remove the two flanking exons. Induction of exons 6 and 8 skipping should result in an in-frame transcript with the splicing of exons 5 to 9. In practise however, targeting exon 8 for removal from the pre-mRNA results in the co-removal of exon 9 so the resultant transcript would have exon 5 joined to exon 10. The inclusion or exclusion of exon 9 does not after the reading frame. Once the antisense molecules to be tested have been identified, they are prepared according to standard techniques known in the art. The most common method for producing antisense molecules is the methylation of the 2' hydroxyribose position and the incorporation of a phosphorothioate backbone produces molecules that superficially resemble RNA but that are much more resistant to nuclease degradation.

[0056] To avoid degradation of pre-mRNA during duplex formation with the antisense molecules, the antisense molecules used in the method may be adapted to minimise or prevent cleavage by endogenous RNase H. This property is highly preferred as the treatment of the RNA with the unmethylated oligonucleotides either intracellularly or in crude extracts that contain RNase H leads to degradation of the pre-mRNA: antisense oligonucleotide duplexes. Any form of modified antisense molecules that is capable of by-passing or not inducing such degradation may be used in the present method. An example of antisense molecules which when duplexed with RNA are not cleaved by cellular RNase H is 2'-O-methyl derivatives. 2'-O-methyl-oligoribonucleotides are very stable in a cellular environment and in animal tissues, and their duplexes with RNA have higher Tm values than their ribo- or deoxyribo- counterparts.

[0057] Antisense molecules that do not activate RNase H can be made in accordance with known techniques (see, e.g., U.S. Pat. 5,149,797). Such antisense molecules, which may be deoxyribonucleotide or ribonucleotide sequences, simply contain any structural modification which sterically hinders or prevents binding of RNase H to a duplex molecule containing the oligonucleotide as one member thereof, which structural modification does not substantially hinder or disrupt duplex formation. Because the portions of the oligonucleotide involved in duplex formation are substantially different from those portions involved in RNase H binding thereto, numerous antisense molecules that do not activate RNase H are available. For example, such antisense molecules may be oligonucleotides wherein at least one, or all, of the inter-nucleotide bridging phosphate residues are modified phosphates, such as methyl phosphonates, methyl phosphorothioates, phosphoromorpholidates, phosphoropiperazidates and phosphoramidates. For example, every other one of the internucleotide bridging phosphate residues may be modified as described. In another nonlimiting example, such antisense molecules are molecules wherein at least one, or all, of the nucleotides contain a 2' lower alkyl moiety (e.g., C_1-C_4, linear or branched, saturated or unsaturated alkyl, such as methyl, ethyl, ethenyl, propyl, 1-propenyl, 2-propenyl, and isopropyl). For example, every other one of the nucleotides may be modified as described.

[0058] While antisense oligonucleotides are a preferred form of the antisense molecules, the present invention comprehends other oligomeric antisense molecules, including but not limited to oligonucleotide mimetics such as are described below.

[0059] Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

[0060] In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleo-bases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone.

[0061] Modified oligonucleotides may also contain one or more substituted sugar moieties. Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. Certain nucleo-bases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

[0062] Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates that enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety, cholic acid, a thioether, e.g., hexyl-S-tritylthiol, a thiocholesterol, an aliphatic chain, e.g., dodecandiol or undecyl residues, a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate, a polyamine or a poly-ethylene glycol chain, or adamantane acetic acid, a palmityl moiety, or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety.

[0063] It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes antisense compounds that are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense molecules, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the increased resistance to nuclease degradation, increased cellular uptake, and an additional region for increased binding affinity for the target nucleic acid.

Methods of Manufacturing Antisense Molecules

[0064] The antisense molecules used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, Calif.). One method for synthesising oligonucleotides on a modified solid support is described in U.S. Pat. No. 4,458,066.

[0065] Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives. In one such automated embodiment, diethyl-phosphoramidites are used as starting materials and may be synthesized as described by Beaucage, et al., (1981) Tetrahedron Letters, 22:1859-1862.

[0066] The antisense molecules of the invention are synthesised *in vitro* and do not include antisense compositions of biological origin, or genetic vector constructs designed to direct the *in vivo* synthesis of antisense molecules. The molecules of the invention may also be mixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption.

Therapeutic Agents

[0067] The present invention also can be used as a prophylactic or therapeutic, which may be utilised for the purpose of treatment of a genetic disease.

[0068] Accordingly, in one embodiment the present invention provides antisense molecules that bind to a selected target In the dystrophin pre-mRNA to Induce efficient and consistent exon skipping described herein in a therapeutically effective amount admixed with a pharmaceutically acceptable carrier, diluent, or excipient.

[0069] The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similarly untoward reaction, such as gastric upset and the like, when administered to a patient. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for Injectable solutions. Suitable pharmaceutical carriers are described In Martin, Remington's Pharmaceutical Sciences, 18th Ed., Mack Publishing Co., Easton, PA, (1990).

[0070] In a more specific form of the invention there are provided pharmaceutical compositions comprising therapeutically effective amounts of an antisense molecule together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content (*e.g.*, Tris-HCl, acetate, phosphate), pH and ionic strength and additives such as detergents and solubilizing agents (e.g., Tween 80, Polysorbate 80), anti-oxidants (*e.g.*, ascorbic acid, sodium metabisulfite), preservatives (*e.g.*, Thimersol, benzyl alcohol) and bulking substances (*e.g.*, lactose, mannitol). The material may be incorporated into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into liposomes. Hylauronic acid may also be used. Such compositions may influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the present proteins and derivatives. *See, e.g.,* Martin, Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435-1712. The compositions may be prepared in liquid form, or may be in dried powder, such as lyophilised form.

[0071] It will be appreciated that pharmaceutical compositions provided according to the present invention may be administered by any means known in the art. Preferably, the pharmaceutical compositions for administration are administered by injection, orally, or by the pulmonary, or nasal route. The antisense molecules are more preferably delivered by intravenous, intra-arterial, intraperitoneal, intramuscular, or subcutaneous routes of administration.

Antisense molecule based therapy

[0072] Also addressed by the present invention is the use of antisense molecules of the present invention, for manufacture of a medicament for modulation of a genetic disease.

[0073] The delivery of a therapeutically useful amount of antisense molecules may be achieved by methods previously published. For example, intracellular delivery of the antisense molecule may be via a composition comprising an admixture of the antisense molecule and an effective amount of a block copolymer. An example of this method is described in US patent application US 20040248833.

[0074] Other methods of delivery of antisense molecules to the nucleus are described in Mann CJ et al., (2001) ["Antisense-induced exon skipping and the synthesis of dystrophin in the mdx mouse". Proc., Natl. Acad. Science, 98 (1) 42-47] and in Gebski et al., (2003). Human Molecular Genetics, 12(15): 1801-1811.

[0075] A method for introducing a nucleic acid molecule into a cell by way of an expression vector either as naked DNA or complexed to lipid carriers, is described in US patent US 6,806,084.

[0076] It may be desirable to deliver the antisense molecule in a colloidal dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes or liposome formulations.

[0077] Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo.* These formulations may have net cationic, anionic or neutral charge characteristics and are useful characteristics with *in vitro, in vivo* and *ex vi*vo delivery methods. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0 .PHI.m can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, and DNA can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, et al., Trends Biochem. Sci., 6:77, 1981).

[0078] In order for a liposome to be an efficient gene transfer vehicle, the following characteristics should be present: (1) encapsulation of the antisense molecule of interest at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino, et al., Biotechniques, 6:682, 1988).

[0079] The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

[0080] Alternatively, the antisense construct may be combined with other pharmaceutically acceptable carriers or diluents to produce a pharmaceutical composition. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular, oral or transdermal administration.

[0081] The routes of administration described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and any dosage for any particular animal and condition.Multiple approaches for introducing functional new genetic material into cells, both in vitro and in vivo have been attempted (Friedmann (1989) Science, 244:1275-1280).

[0082] These approaches include integration of the gene to be expressed into modified retroviruses (Friedmann (1989) supra; Rosenberg (1991) Cancer Research 51(18), suppl.: 5074S-5079S); integration into non-retrovirus vectors (Rosenfeld, et al. (1992) Cell, 68:143-155; Rosenfeld, et al. (1991) Science, 252:431-434); or delivery of a transgene linked to a heterologous promoter-enhancer element via liposomes (Friedmann (1989), supra; Brigham, et al. (1989) Am. J. Med. Sci., 298:278-281; Nabel, et al. (1990) Science, 249:1285-1288; Hazinski, et al. (1991) Am. J. Resp. Cell Molec. Biol., 4:206-209; and Wang and Huang (1987) Proc. Natl. Acad. Sci. (USA), 84:7851-7855); coupled to ligand-specific, cation-based transport systems (Wu and Wu (1988) J. Biol. Chem., 263:14621-14624) or the use of naked DNA, expression vectors (Nabel et al. (1990), supra); Wolff et al. (1990) Science, 247:1465-1468). Direct injection of transgenes into tissue produces only localized expression (Rosenfeld (1992) supra); Rosenfeld et al. (1991) supra; Brigham et al. (1989) supra; Nabel (1990) supra; and Hazinski et al. (1991) supra). The Brigham et al. group (Am. J. Med. Sci. (1989) 298: 278-281 and Clinical Research (1991) 39 (abstract)) have reported in vivo transfection only of lungs of mice following either intravenous or intratracheal administration of a DNA liposome complex. An example of a review article of human gene therapy procedures is: Anderson, Science (1992) 256:808-813.

[0083] The antisense molecules of the invention encompass any pharmaceutically acceptable salts, esters, or salts

of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such pro-drugs, and other bioequivalents.

[0084] The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

[0085] For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, (including by nebulizer, intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intra-arterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

[0086] The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Kits of the Invention

[0087] The invention also provides kits for treatment of a patient with a genetic disease which kit comprises at least an antisense molecule, packaged in a suitable container, together with instructions for its use.

[0088] In a preferred embodiment, the kits will contain at least one antisense molecule. The kits may also contain peripheral reagents such as buffers, stabilizers, etc.

[0089] Those of ordinary skill in the field should appreciate that applications of the above method has wide application for identifying antisense molecules suitable for use in the treatment of many other diseases.

EXAMPLES

[0090] The following Examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these Examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes.

[0091] Methods of molecular cloning, immunology and protein chemistry, which are not explicitly described in the following examples, are reported In the literature and are known by those skilled in the art. General texts that described conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art, included, for example: Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989); Glover ed., DNA Cloning: A Practical Approach, Volumes I and II, MRL Press, Ltd., Oxford, U.K. (1985); and Ausubel, F., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. Current Protocols In Molecular Biology. Greene Publishing Associates/Wiley Intersciences, New York (2002).

Determining Induced Exon Skipping in Human Muscle Cells

[0092] Attempts by the inventors to develop a rational approach in antisense molecules design were not completely successful as there did not appear to be a consistent trend that could be applied to all exons. As such, the identification of the most effective and therefore most therapeutic antisense molecules compounds has been the result of empirical studies.

[0093] These empirical studies involved the use of computer programs to identify motifs potentially involved in the splicing process. Other computer programs were also used to identify regions of the pre-mRNA which may not have

had extensive secondary structure and therefore potential sites for annealing of antisense molecules. Neither of these approaches proved completely reliable in designing antisense oligonucleotides for reliable and efficient induction of exon skipping.

[0094] Annealing sites on the human dystrophin pre-mRNA were selected for examination, initially based upon known or predicted motifs or regions involved in splicing. 2OMe antisense oligonucleotides were designed to be complementary to the target sequences under investigation and were synthesised on an Expedite 8909 Nucleic Acid Synthesiser. Upon completion of synthesis, the oligonucleotides were cleaved from the support column and de-protected in ammonium hydroxide before being desalted. The quality of the oligonucleotide synthesis was monitored by the intensity of the trityl signals upon each deprotection step during the synthesis as detected in the synthesis log. The concentration of the antisense oligonucleotide was estimated by measuring the absorbance of a diluted aliquot at 260nm.

[0095] Specified amounts of the antisense molecules were then tested for their ability to induce exon skipping in an *in vitro* assay, as described below.

[0096] Briefly, normal primary myoblast cultures were prepared from human muscle biopsies obtained after informed consent. The cells were propagated and allowed to differentiate into myotubes using standard culturing techniques. The cells were then transfected with the antisense oligonucleotides by delivery of the oligonucleotides to the cells as cationic lipoplexes, mixtures of antisense molecules or cationic liposome preparations.

[0097] The cells were then allowed to grow for another 24 hours, after which total RNA was extracted and molecular analysis commenced. Reverse transcriptase amplification (RT-PCR) was undertaken to study the targeted regions of the dystrophin pre-mRNA or induced exonic re-arrangements.

[0098] For example, in the testing of an antisense molecule for inducing exon 19 skipping the RT-PCR test scanned several exons to detect Involvement of any adjacent exons. For example, when inducing skipping of exon 19, RT-PCR was carried out with primers that amplified across exons 17 and 21. Amplifications of even larger products in this area (i.e. exons 13-26) were also carried out to ensure that there was minimal amplification bias for the shorter induced skipped transcript. Shorter or exon skipped products tend to be amplified more efficiently and may bias the estimated of the normal and induced transcript.

[0099] The sizes of the amplification reaction products were estimated on an agarose gel and compared against appropriate size standards. The final confirmation of identity of these products was carried out by direct DNA sequencing to establish that the correct or expected exon junctions have been maintained.

[0100] Once efficient exon skipping had been induced with one antisense molecule, subsequent overlapping antisense molecules may be synthesized and then evaluated in the assay as described above. Our definition of an efficient antisense molecule is one that induces strong and sustained exon skipping at transfection concentrations in the order of 300 nM or less.

Antisense Oligonucleotides Directed at Exon 51

[0101] Antisense oligonucleotides directed at exon 51 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

[0102] Antisense oligonucleotide H51A(+66+90) [SEQ ID NO:180] showed the stronger ability to Induce exon 51 skipping. Table 1 below includes antisense molecules tested at a concentration range of 25, 50, 100, 300 and 600 nM. These antisense molecules showed varying ability to induce exon 51 skipping. The strongest inducers of exon skipping were antisense oligonucleotide H51A(+61+90) [SEQ ID NO: 179] and H51A(+66+95) [SEQ ID NO: 181].

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H51A(-01+25)	ACC AGA GUA ACA GUC UGA GUA GGA GC	Faint skipping
H51D(+16-07)	CUC AUA CCU UCU GCU UGA UGA UC	Skipping at 300 nM
H51A(+111+134)	UUC UGU CCA AGC CCG GUU GAA AUC	Needs retesting
H51A(+61+90)	ACA UCA AGG AAG AUG GCA UUU CUA GUU UGG	Very strong skipping
H51A(+66+90)	ACA UCA AGG AAG AUG GCA UUU CUA G	skipping
H51A(+66+95)	CUC CAA CAU CAA GGA AGA UGG CAU UUC UAG	Very strong skipping
H51D(+08-17)	AUC AUU UUU UCU CAU ACC UUC UGC U	No skipping

(continued)

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H51A/D(+08-17) & (-15+?)	AUC AUU UUU UCU CAU ACC UUC UGC UAG GAG CUA AAA	No skipping
H51A(+175+195)	CAC CCA CCA UCA CCC UCY GUG .	No skipping
H51A(+199+220)	AUC AUC UCG UUG AUA UCC UCA A	No skipping

SEQUENCE LISTING

[0103]

<110> The university of Western Australia

<120> Antisense oligonucleotides for Inducing Exon skipping and Methods of use Thereof.

<130> 111819

<160> 211

<170> PatentIn version 3.3

<210> 1
<211> 24
<212> DNA
<213> Human

<400> 1
gauagguggu aucaacaucu guaa 24

<210> 2
<211> 21
<212> DNA
<213> human

<400> 2
gauagguggu aucaacaucu g 21

<210> 3
<211> 25
<212> DNA
<213> human

<400> 3
gauagguggu aucaacaucu guaag 25

<210> 4
<211> 20
<212> DNA
<213> human

<400> 4
ggugguauca acaucuguaa 20

<210> 5

<211> 20
<212> DNA
<213> human

<400> 5
guaucaacau cuguaagcac 20

<210> 6
<211> 23
<212> DNA
<213> human

<400> 6
ugcauguucc agucguugug ugg 23

<210> 7
<211> 25
<212> DNA
<213> human

<400> 7
cacuauucca gucaaauagg ucugg 25

<210> 8
<211> 25
<212> DNA
<213> human

<400> 8
auuuaccaac cuucaggauc gagua 25

<210> 9
<211> 21
<212> DNA
<213> human

<400> 9
ggccuaaaac acauacacau a 21

<210> 10
<211> 20
<212> DNA
<213> human

<400> 10
cauuuuugac cuacaugugg 20

<210> 11
<211> 20
<212> DNA
<213> human

<400> 11
uuugaccuac auguggaaag 20

<210> 12
<211> 26
<212> DNA

<213> human

<400> 12
uacauuuuug accuacaugu ggaaag 26

<210> 13
<211> 22
<212> DNA
<213> human

<400> 13
auuuuugacc uacaugggaa ag 22

<210> 14
<211> 23
<212> DNA
<213> human

<400> 14
uacgaguuga uugucggacc cag 23

<210> 15
<211> 25
<212> DNA
<213> human

<400> 15
guggucuccu uaccuaugac ugugg 25

<210> 16
<211> 17
<212> DNA
<213> human

<400> 16
ggucuccuua ccuauga 17

<210> 17
<211> 24
<212> DNA
<213> human

<400> 17
ugucucagua aucuucuuac cuau 24

<210> 18
<211> 24
<212> DNA
<213> human

<400> 18
ucuuaccuau gacuauggau gaga 24

<210> 19
<211> 20
<212> DNA
<213> human

<400> 19
gcaugaacuc uuguggaucc 20

<210> 20
<211> 20
<212> DNA
<213> human

<400> 20
ccaggguacu acuuacauua 20

<210> 21
<211> 21
<212> DNA
<213> human

<400> 21
aucgugugue acagcaucca g 21

<210> 22
<211> 30
<212> DNA
<213> human

<400> 22
uguucagggc augaacucuu guggauccuu 30

<210> 23
<211> 31
<212> DNA
<213> human

<400> 23
uaggaggcgc cucccauccu guaggucacu g 31

<210> 24
<211> 31
<212> DNA
<213> human

<400> 24
aggucuagga ggcgccuccc auccuguagg u 31

<210> 25 .
<211> 25
<212> DNA
<213> human

<400> 25
gcgccuccca uccuguaggu cacug 25

<210> 26
<211> 26
<212> DNA
<213> human

<400> 26
cuucgaggag gucuaggagg cgccuc 26

<210> 27
<211> 21
<212> DNA
<213> human

<400> 27
cucccauccu guaggucacu g 21

<210> 28
<211> 22
<212> DNA
<213> human

<400> 28
uaccaguuuu ugcccuguca gg 22

<210> 29
<211> 26
<212> DNA
<213> human

<400> 29
ucaauaugcu gcuucccaaa cugaaa 26
<210> 30
<211> 25
<212> DNA
<213> human

<400> 30
cuaggaggcg ccucccaucc uguag 25

<210> 31
<211> 31
<212> DNA
<213> human

<400> 31
uuaugauuuc caucuacgau gucaguacuu c 31

<210> 32
<211> 31
<212> DNA
<213> human

<400> 32
cuuaccugcc aguggaggau uauauuccaa a 31

<210> 33
<211> 25
<212> DNA
<213> human

<400> 33
caucaggauu cuuaccugcc agugg 25

<210> 34
<211> 25
<212> DNA

<213> human

<400> 34
cgaugucagu acuuccaaua uucac 25

<210> 35
<211> 18
<212> DNA
<213> human

<400> 35
accauucauc aggauucu 18

<210> 36
<211> 18
<212> DNA
<213> human

<400> 36
accugccagu ggaggauu 18

<210> 37
<211> 27
<212> DNA
<213> human

<400> 37
ccaauauuca cuaaaucaac cuguuaa 27

<210> 38
<211> 30
<212> DNA
<213> human

<400> 38
caggauucuu accugccagu ggaggauuau 30

<210> 39
<211> 31
<212> DNA
<213> human

<400> 39
acgaugucag uacuuccaau auucacuaaa u 31

<210> 40
<211> 31
<212> DNA
<213> human

<400> 40
auuuccaucu acgaugucag uacuuccaau a 31

<210> 41
<211> 21
<212> DNA
<213> human

<400> 41
caggagcuuc caaaugcugc a 21

<210> 42
<211> 29
<212> DNA
<213> human

<400> 42
cuugucuuca ggagcuucca aaugcugca 29

<210> 43
<211> 22
<212> DNA
<213> human

<400> 43
uccucagcag aaagaagcca cg 22

<210> 44
<211> 20
<212> DNA
<213> human

<400> 44
uuagaaaucu cuccuugugc 20

<210> 45
<211> 20
<212> DNA
<213> human

<400> 45 5
uaaauugggu guuacacaau 20

<210> 46
<211> 24
<212> DNA
<213> human

<400> 46
cccugaggca uucccaucuu gaau 24

<210> 47
<211> 20
<212> DNA
<213> human

<400> 47
aggacuuacu ugcuuuguuu 20

<210> 48
<211> 23
<212> DNA
<213> human

<400> 48
cuugaauuua ggagauucau cug 23

<210> 49
<211> 23
<212> DNA
<213> human

<400> 49
caucuucuga uaauuuuccu guu 23

<210> 50
<211> 24
<212> DNA
<213> human

<400> 50
ucuucuguuu uuguuagcca guca 24

<210> 51
<211> 20
<212> DNA
<213> human

<400> 51
ucuauguaaa cugaaaauuu 20

<210> 52
<211> 20
<212> DNA
<213> human

<400> 52
uucuggagau ccauuaaaac 20

<210> 53
<211> 24
<212> DNA
<213> human

<400> 53
cagcaguugc gugaucucca cuag 24

<210> 54
<211> 21
<212> DNA
<213> human

<400> 54
uucaucaacu accaccacca u 21

<210> 55
<211> 25
<212> DNA
<213> human

<400> 55
cuaagcaaaa uaaucugacc uuaag 25

<210> 56
<211> 28

<212> DNA
<213> human

<400> 56
cuuguaaaag aacccagcgg ucuucugu 28

<210> 57
<211> 22
<212> DNA
<213> human

<400> 57
caucuacaga uguuugccca uc 22

<210> 58
<211> 23
<212> DNA
<213> human

<400> 58
gaaggauguc uuguaaaaga acc 23

<210> 59
<211> 20
<212> DNA
<213> human

<400> 59
accuguucuu caguaagacg 20

<210> 60
<211> 24
<212> DNA
<213> human

<400> 60
caugacacac cuguucuuca guaa 24

<210> 61
<211> 20
<212> DNA
<213> human

<400> 61
cauuugagaa ggaugucuug 20

<210> 62
<211> 24
<212> DNA
<213> human

<400> 62
aucucccaau accuggagaa gaga 24

<210> 63
<211> 31
<212> DNA
<213> human

<400> 63
gccaugcacu aaaaaggcac ugcaagacau u 31

<210> 64
<211> 24
<212> DNA
<213> human

<400> 64
ucuuuaaagc caguugugug aauc 24

<210> 65
<211> 21
<212> DNA
<213> human

<400> 65
uuucugaaag ccaugcacua a 21

<210> 66
<211> 25
<212> DNA
<213> human

<400> 66
guacauacgg ccaguuuuug aagac 25

<210> 67
<211> 31
<212> DNA
<213> human

<400> 67
cuagauccgc uuuuaaaacc uguuaaaaca a 31

<210> 68
<211> 31
<212> DNA
<213> human

<400> 68
ucuuuucuag auccgcuuuu aaaaccuguu a 31

<210> 69
<211> 25
<212> DNA
<213> human

<400> 69
cuagauccgc uuuuaaaacc uguua 25

<210> 70
<211> 23
<212> DNA
<213> human

<400> 70
ccgucuucug ggucacugac uua 23

<210> 71
<211> 26
<212> DNA
<213> human

<400> 71
cuagauccgc uuuuaaaacc uguuaa 26

<210> 72
<211> 20
<212> DNA
<213> human

<400> 72
ccgcuuuuaa aaccuguuaa 20

<210> 73
<211> 26
<212> DNA
<213> human

<400> 73
uggauugcuu uucuuuucu agaucc 26

<210> 74
<211> 25
<212> DNA
<213> human

<400> 74
caugcuuccg ucuucugggu cacug 25

<210> 75
<211> 23
<212> DNA
<213> human

<400> 75
gaucuuguuu gagugaauac agu 23

<210> 76
<211> 22
<212> DNA
<213> human

<400> 76
guuauccagc caugcuuccg uc 22

<210> 77
<211> 25
<212> DNA
<213> human

<400> 77
ugauaauugg uaucacuaac cugug 25

<210> 78
<211> 22

<212> DNA
<213> human

<400> 78
guaucacuaa ccugugcugu ac 22

<210> 79
<211> 20
<212> DNA
<213> human

<400> 79
cagcaguagu ugucaucugc 20

<210> 80
<211> 31
<212> DNA
<213> human

<400> 80
gccugagcug aucugcuggc aucuugcagu u 31

<210> 81
<211> 28
<212> DNA
<213> human

<400> 81
cuggcagaau ucgauccacc ggcuguuc 28

<210> 82
<211> 22
<212> DNA
<213> human

<400> 82
cagcaguagu ugucaucugc uc 22

<210> 83
<211> 19
<212> DNA
<213> human

<400> 83
ugauggggug guggguugg 19

<210> 84
<211> 25
<212> DNA
<213> human

<400> 84
aucugcauua acacccucua gaaag 25

<210> 85
<211> 24
<212> DNA
<213> human

<400> 85
ccggcuguuc aguuguucug aggc 24

<210> 86
<211> 28
<212> DNA
<213> human

<400> 86
aucugcauua acacccucua gaaagaaa 28

<210> 87
<211> 28
<212> DNA
<213> human

<400> 87
gaaggagaag agauucuuac cuuacaaa 28

<210> 88
<211> 20
<212> DNA
<213> human

<400> 88
auucgaucca ccggcuguuc 20

<210> 89
<211> 19
<212> DNA
<213> human

<400> 89
cugcuggcau cuugcaguu 19

<210> 90
<211> 22
<212> DNA
<213> human

<400> 90
gccgguugac uucauccugu gc 22

<210> 91
<211> 22
<212> DNA
<213> human

<400> 91
cugcauccag gaacaugggu cc 22

<210> 92
<211> 23
<212> DNA
<213> human

<400> 92
gucugcaucc aggaacaugg guc 23

<210> 93
<211> 24
<212> DNA
<213> human

<400> 93
guugaagauc ugauagccgg uuga 24

<210> 94
<211> 24
<212> DNA
<213> human

<400> 94
uacuuacugu cuguagcucu uucu 24

<210> 95
<211> 24
<212> DNA
<213> human

<400> 95
cacucauggu cuccugauag cgca 24

<210> 96
<211> 22
<212> DNA
<213> human

<400> 96
cugcaauucc ccgagucucu gc 22

<210> 97
<211> 23
<212> DNA
<213> human

<400> 97
acugcuggac ccauguccug aug 23

<210> 98
<211> 21
<212> DNA
<213> human

<400> 98
cuaaguugag guauggagag u 21

<210> 99
<211> 23
<212> DNA
<213> human

<400> 99
uauucacaga ccugcaauuc ccc 23

<210> 100
<211> 26

<212> DNA
<213> human

<400> 100
acaguggugc ugagauagua uaggcc 26

<210> 101
<211> 22
<212> DNA
<213> human

<400> 101
uaggccacuu uguugcucuu gc 22

<210> 102
<211> 19
<212> DNA
<213> human

<400> 102
uucagagggc gcuuucuuc 19

<210> 103
<211> 23
<212> DNA
<213> human

<400> 103
gggcaggcca uuccuccuuc aga 23

<210> 104
<211> 24
<212> DNA
<213> human

<400> 104
ucuucagggu uuguauguga uucu 24

<210> 105
<211> 27
<212> DNA
<213> human

<400> 105
cugggcugaa uugucugaau aucacug 27

<210> 106
<211> 26
<212> DNA
<213> human

<400> 106
cuguuggcac augugauccc acugag 26

<210> 107
<211> 24
<212> DNA
<213> human

<400> 107
gucuauaccu guuggcacau guga 24

<210> 108
<211> 25
<212> DNA
<213> human

<400> 108
ugcuuucugu aauucaucug gaguu 25

<210> 109
<211> 26
<212> DNA
<213> human

<400> 109
ccuccuuucu ggcauagacc uuccac 26

<210> 110
<211> 25
<212> DNA
<213> human

<400> 110
ugugucaucc auucgugcau cucug 25

<210> 111
<211> 25
<212> DNA
<213> human

<400> 111
uuaaggccuc uugugcuaca ggugg 25

<210> 112
<211> 23
<212> DNA
<213> human

<400> 112
gggccucuuc uuuagcucuc uga 23

<210> 113
<211> 22
<212> DNA
<213> human

<400> 113
gacuuccaaa gucuugcauu uc 22

<210> 114
<211> 24
<212> DNA
<213> human

<400> 114
gccaacaugc ccaaacuucc uaag 24

<210> 115
<211> 26
<212> DNA
<213> human

<400> 115
cagagauuuc cucagcuccg ccagga 26

<210> 116
<211> 21
<212> DNA
<213> human

<400> 116
cuuaraucua gcaccucaga g 21

<210> 117
<211> 25
<212> DNA
<213> human

<400> 117
uccgccaucu guuagggucu gugcc 25

<210> 118
<211> 25
<212> DNA
<213> human

<400> 118
auuuggguua uccucugaau gucgc 25

<210> 119
<211> 22
<212> DNA
<213> human

<400> 119
cauaccucuu cauguaguuc uc 22

<210> 120
<211> 31
<212> DNA
<213> human

<400> 120
cauuugagcu gcguccaccu ugucgucugu g 31

<210> 121
<211> 26
<212> DNA
<213> human

<400> 121
uccugggcag acuggaugcu cuguuc 26

<210> 122
<211> 23

<212> DNA
<213> human

<400> 122
uugccugggc uuccugaggc auu 23

<210> 123
<211> 24
<212> DNA
<213> human

<400> 123
uucugaaaua acauauaccu gugc 24

<210> 124
<211> 25
<212> DNA
<213> human

<400> 124
uaguuucuga aauaacauau accug 25

<210> 125
<211> 21
<212> DNA
<213> human

<400> 125
gacuugucaa aucagauugg a 21

<210> 126
<211> 24
<212> DNA
<213> human

<400> 126
guuucugaaa uaacauauac cugu 24

<210> 127
<211> 20
<212> DNA
<213> human

<400> 127
caccagaaau acauaccaca 20

<210> 128
<211> 20
<212> DNA
<213> human

<400> 128
caaugauuua gcugugacug 20

<210> 129
<211> 23
<212> DNA
<213> human

<400> 129
cgaaacuuca uggagacauc uug 23

<210> 130
<211> 25
<212> DNA
<213> human

<400> 130
cuuguagacg cugcucaaaa uuggc 25

<210> 131
<211> 20
<212> DNA
<213> human

<400> 131 20
caugcacaca ccuuugcucc 20

<210> 132
<211> 24
<212> DNA
<213> human

<400> 132
ucuguacaau cugacgucca gucu 24

<210> 133
<211> 27
<212> DNA
<213> human

<400> 133
gucuuuauca ccauuuccac uucagac 27

<210> 134
<211> 25
<212> DNA
<213> human

<400> 134
ccgucugcuu uuucuguaca aucug 25

<210> 135
<211> 22
<212> DNA
<213> human

<400> 135
uccauaucug uagcugccag cc 22

<210> 136
<211> 23
<212> DNA
<213> human

<400> 136
ccaggcaacu ucagaaucca aau 23

<210> 137
<211> 30
<212> DNA
<213> human

<400> 137
uuucuguuac cugaaaagaa uuauaaugaa 30

<210> 138
<211> 25
<212> DNA
<213> human

<400> 138
cauucauuuc cuuucgcauc uuacg 25

<210> 139
<211> 26
<212> DNA
<213> human

<400> 139
ugaucucuuu gucaauucca uaucug 26

<210> 140
<211> 27
<212> DNA
<213> human

<400> 140
uucagugaua uagguuuuac cuuuccc 27

<210> 141
<211> 26
<212> DNA
<213> human

<400> 141
cuguagcugc cagccauucu gucaag 26

<210> 142
<211> 21
<212> DNA
<213> human

<400> 142
ucuucugcuc gggaggugac a 21

<210> 143
<211> 20
<212> DNA
<213> human

<400> 143 20
ccaguuacua uucagaagac 20

<210> 144
<211> 20

<212> DNA
<213> human

<400> 144
ucuucaggug caccuucugu 20

<210> 145
<211> 25
<212> DNA
<213> human

<400> 145
ugugaugugg uccacauucu gguca 25

<210> 146
<211> 20
<212> DNA
<213> human

<400> 146
ccauguguuu cugguauucc 20

<210> 147
<211> 25
<212> DNA
<213> human

<400> 147
cguguagagu ccaccuuugg gcgua 25

<210> 148
<211> 24
<212> DNA
<213> human

<400> 148
uacuaauuuc cugcaguggu cacc 24

<210> 149
<211> 24
<212> DNA
<213> human

<400> 149
uucuguguga aauggcugca aauc 24

<210> 150
<211> 20
<212> DNA
<213> human

<400> 150
ccuucaaagg aauggaggcc 20

<210> 151
<211> 25
<212> DNA
<213> human

<400> 151
ugcugaauuu cagccuccag ugguu 25

<210> 152
<211> 25
<212> DNA
<213> human

<400> 152
ugaagucuuc cucuuucaga uucac 25

<210> 153
<211> 24
<212> DNA
<213> human

<400> 153
cuggcuuucu cucaucugug auuc 24

<210> 154
<211> 20
<212> DNA
<213> human

<400> 154
guuguaaguu gucuccucuu 20

<210> 155
<211> 20
<212> DNA
<213> human

<400> 155
uugucuguaa cagcugcugu 20

<210> 156
<211> 20
<212> DNA
<213> human

<400> 156
gcucuaauac cuugagagca 20

<210> 157
<211> 22
<212> DNA
<213> human

<400> 157
cuuugagacc ucaaauccug uu 22

<210> 158
<211> 25
<212> DNA
<213> human

<400> 158
cuuuauuuuc cuuucaucuc ugggc 25

<210> 159
<211> 27
<212> DNA
<213> human

<400> 159
aucguuucuu cacggacagu gugcugg 27

<210> 160
<211> 24
<212> DNA
<213> human

<400> 160
gggcuuguga gacaugagug auuu 24

<210> 161
<211> 22
<212> DNA
<213> human

<400> 161
accuucagag gacuccucuu gc 22

<210> 162
<211> 25
<212> DNA
<213> human

<400> 162
uauguguuac cuacccuugu cgguc 25.

<210> 163
<211> 20
<212> DNA
<213> human

<400> 163
ggagagagcu uccuguagcu 20

<210> 164
<211> 23
<212> DNA
<213> human

<400> 164
ucacccuuuc cacaggcguu gca 23

<210> 165
<211> 20
<212> DNA
<213> human

<400> 165
uuugugucuu ucugagaaac 20

<210> 166
<211> 20

<212> DNA
<213> human

<400> 166
aaagacuuac cuuaagauac 20

<210> 167
<211> 20
<212> DNA
<213> human

<400> 167
aucugucaaa ucgccugcag 20

<210> 168
<211> 20
<212> DNA
<213> human

<400> 168
uuaccuugac uugcucaagc 20

<210> 169
<211> 20
<212> DNA
<213> human

<400> 169
uccagguuca agugggauac 20

<210> 170
<211> 25
<212> DNA
<213> human

<400> 170
gcucuucugg gcuuauggga gcacu 25

<210> 171
<211> 27
<212> DNA
<213> human

<400> 171
accuuuaucc acuggagauu ugucugc 27

<210> 172
<211> 21
<212> DNA
<213> human

<400> 172
uuccaccagu aacugaaaca g 21

<210> 173
<211> 29
<212> DNA
<213> human

<400> 173
ccacucagag cucagaucuu cuaacuucc 29

<210> 174
<211> 27
<212> DNA
<213> human

<400> 174
cuucaacuca gagcucagau cuucuaa 27

<210> 175
<211> 25
<212> DNA
<213> human

<400> 175
gggauccagu auacuuacag gcucc 25

<210> 176
<211> 26
<212> DNA
<213> human

<400> 176
accagaguaa cagucugagu aggagc 26

<210> 177
<211> 23
<212> DNA
<213> human

<400> 177
cucauaccuu cugcuugaug auc 23

<210> 178
<211> 24
<212> DNA
<213> human

<400> 178
uucuguccaa gcccgguuga aauc 24

<210> 179
<211> 25
<212> DNA
<213> human

<400> 179
acaucaagga ggauggcauu ucuag 25

<210> 180
<211> 25
<212> DNA
<213> human

<400> 180
acaucaagga agauggcauu ucuag 25

<210> 181
<211> 30
<212> DNA
<213> human

<400> 181
cuccaacauc aaggaagaug gcauuucuag 30

<210> 182
<211> 25
<212> DNA
<213> human

<400> 182
aucauuuuuu cucauaccuu cugcu 25

<210> 183
<211> 36
<212> DNA
<213> human

<400> 183
aucauuuuuu cucauaccuu cugcuaggag cuaaaa 36

<210> 184
<211> 21
<212> DNA
<213> human

<400> 184
cacccaccau cacccucugu g 21

<210> 185
<211> 22
<212> DNA
<213> human

<400> 185
aucaucucgu ugauauccuc aa 22

<210> 186
<211> 21
<212> DNA
<213> human

<400> 186
uccugcauug uugccuguaa g 21

<210> 187
<211> 30
<212> DNA
<213> human

<400> 187
uccaacuggg gacgccucug uuccaaaucc 30

<210> 188
<211> 21

<212> DNA
<213> human

<400> 188
acuggggacg ccucuguucc a 21

<210> 189
<211> 20
<212> DNA
<213> human

<400> 189
ccguaaugau uguucuagcc 20

<210> 190
<211> 20
<212> DNA
<213> human

<400> 190
uguuaaaaaa cuuacuucga 20

<210> 191
<211> 31
<212> DNA
<213> human

<400> 191
cauucaacug uugccuccgg uucugaaggu g 31

<210> 192
<211> 24
<212> DNA
<213> human

<400> 192
cuguugccuc cgguucugaa ggug 24

<210> 193
<211> 25
<212> DNA
<213> human

<400> 193
cauucaacug uugccuccgg uucug 25

<210> 194
<211> 21
<212> DNA
<213> human

<400> 194 21
uacuaaccuu gguuucugug a 21

<210> 195
<211> 25
<212> DNA
<213> human

<400> 195
cugaaggugu ucuuguacuu caucc 25

<210> 196
<211> 27
<212> DNA
<213> human

<400> 196
uguauaggga cccuccuucc augacuc 27

<210> 197
<211> 25
<212> DNA
<213> human

<400> 197
cuaaccuugg uuucugugau uuucu 25

<210> 198
<211> 27
<212> DNA
<213> human

<400> 198
gguaucuuug auacuaaccu ugguuuc 27

<210> 199
<211> 22
<212> DNA
<213> human

<400> 199
auucuuucaa cuagaauaaa ag 22

<210> 200
<211> 25
<212> DNA
<213> human

<400> 200
gauucugaau ucuuucaacu agaau 25

<210> 201
<211> 20
<212> DNA
<213> human

<400> 201
aucccacuga uucugaauuc 20

<210> 202
<211> 22
<212> DNA
<213> human

<400> 202
uuggcucugg ccuguccuaa ga 22

<210> 203
<211> 30
<212> DNA
<213> human

<400> 203
cucuuuucca gguucaagug ggauacuagc 30

<210> 204
<211> 31
<212> DNA
<213> human

<400> 204
caagcuuuuc uuuuaguugc ugcucuuuuc c 31

<210> 205
<211> 30
<212> DNA
<213> human

<400> 205
uauucuuuug uucuucuagc cuggagaaag 30

<210> 206
<211> 28
<212> DNA
<213> human

<400> 206
cugcuucccuc caaccauaaa acaaauuc 28

<210> 207
<211> 26
<212> DNA
<213> human

<400> 207
ccaaugccau ccuggaguuc cuguaa 26

<210> 208
<211> 20
<212> DNA
<213> human

<400> 208
uccuguagaa uacuggcauc 20

<210> 209
<211> 27
<212> DNA
<213> human

<400> 209
ugcagaccuc cugccaccgc agauuca 27

<210> 210
<211> 20

<212> DNA
<213> human

<400> 210
cuaccucuuu uuucugucug 20

<210> 211

<211> 20
<212> DNA
<213> human

<400> 211
uguuuuugag gauugcugaa 20

Claims

1. An antisense oligonucleotide that binds to the human dystrophin gene to induce exon skipping in the dystrophin gene, consisting of the sequence of SEQ ID NO: 181, optionally wherein the uracil bases (U) are thymine bases (T).

2. An isolated antisense oligonucleotide of up to 50 nucleotides in length, wherein the antisense oligonucleotide comprises the antisense oligonucleotide of claim 1.

3. The antisense oligonucleotide of claim 1, wherein the antisense oligonucleotide comprises a modified backbone or non-natural internucleotide linkages.

4. The antisense oligonucleotide of claim 1, wherein the antisense oligonucleotide is chemically linked to one or more moieties or conjugates that enhance the activity, cellular distribution, or cellular uptake of the antisense oligonucleotide.

5. The antisense oligonucleotide of claim 1, wherein the antisense oligonucleotide does not activate RNase H.

6. The antisense oligonucleotide of claim 3, wherein the sugar moieties of the oligonucleotide backbone are replaced with non-natural moieties.

7. The antisense oligonucleotide of claim 3, wherein the modified backbone comprises morpholinos.

8. The antisense oligonucleotide of claim 1, wherein the inter-nucleotide linkages of the oligonucleotide backbone are replaced with non-natural inter-nucleotide linkages.

9. The antisense oligonucleotide of claim 8, wherein the non-natural inter-nucleotide linkages are modified phosphates.

10. The antisense oligonucleotide of claim 9, wherein the modified phosphates are selected from methyl phosphonates, methyl phosphorothioates, phosphoromorpholidates, phosphoropiperazidates, and phosphoroamidates.

11. The antisense oligonucleotide of claim 10, wherein the modified phosphates are selected from phosphoroamidates.

12. The antisense oligonucleotide of claim 10 wherein the modified phosphates are selected from phosphoromorpholidates.

13. The antisense oligonucleotide of claim 10, wherein the modified phosphates are selected from phosphoropiperazidates.

14. The antisense oligonucleotide of claim 1, wherein the sugar moieties of the oligonucleotide backbone are replaced with non-natural moieties and the inter-nucleotide linkages of the oligonucleotide backbone are replaced with non-natural inter-nucleotide linkages.

15. The antisense oligonucleotide of any of claims 1-14, wherein the uracil bases are thymine bases.

16. The antisense oligonucleotide of claim 15, wherein the antisense oligonucleotide is chemically linked to one or more moieties or conjugates that enhance the activity, cellular distribution, or cellular uptake of the antisense oligonucleotide.

17. The antisense oligonucleotide of claim 16, wherein the antisense oligonucleotide is chemically linked to a polyethylene glycol chain.

18. A composition comprising an antisense oligonucleotide according to any one of the preceding claims and one or more pharmaceutically acceptable carriers and/or diluents.

19. An antisense oligonucleotide according to any one of claims I to 17 or a composition according to claim 18 for use in a method of treatment of muscular dystrophy in a patient.

Patentansprüche

1. Ein Antisense-Oligonukleotid, das an humanes Dystrophin-Gen bindet, um das Überspringen von Exons in dem Dystrophin-Gen zu induzieren, das aus der Sequenz mit SEQ ID NO: 181 besteht, wobei optional die Uracil-Basen (U) Thymin-Basen (T) sind.

2. Ein isoliertes Antisense-Oligonukleotid mit einer Länge von bis zu 50 Nukleotiden, wobei das Antisense-Oligonukleotid das Antisense-Oligonukleotid nach Anspruch 1 umfasst.

3. Das Antisense-Oligonukleotid nach Anspruch 1, wobei das Antisense-Oligonukleotid ein modifiziertes Rückgrat oder unnatürliche Inter-Nukleotid-Bindungen umfasst.

4. Das Antisense-Oligonukleotid nach Anspruch 1, wobei das Antisense-Oligonukleotid chemisch an eine oder mehrere Einheiten oder Konjugate gebunden ist, die die Aktivität, die zelluläre Verteilung oder die zelluläre Aufnahme des Antisense-Oligonukleotids verstärken.

5. Das Antisense-Oligonukleotid nach Anspruch 1, wobei das Antisense-Oligonukleotid RNase H nicht aktiviert.

6. Das Antisense-Oligonukleotid nach Anspruch 3, wobei die Zucker-Einheiten des Oligonukleotid-Rückgrats mit unnatürlichen Einheiten ersetz werden.

7. Das Antisense-Oligonukleotid nach Anspruch 3, wobei das modifizierte Rückgrat Morpholinos umfasst.

8. Das Antisense-Oligonukleotid nach Anspruch 1, wobei die Inter-Nukleotid-Bindungen des Oligonukleotid-Rückgrats mit unnatürlichen Inter-Nukleotid-Bindungen ersetzt werden.

9. Das Antisense-Oligonukleotid nach Anspruch 8, wobei die unnatürlichen Inter-Nukleotid-Bindungen modifizierte Phosphate sind.

10. Das Antisense-Oligonukleotid nach Anspruch 9, wobei die modifizierten Phosphate aus Methylphosphonaten, Methylphosporothioaten, Phosphoromorpholidaten, Phosphoropiperazidaten und Phosphoroamidaten ausgewählt werden.

11. Das Antisense-Oligonukleotid nach Anspruch 10, wobei die modifizierten Phosphate aus Phosphoroamidaten ausgewählt werden.

12. Das Antisense-Oligonukleotid nach Anspruch 10, wobei die modifizierten Phosphate aus Phosphoromorpholidaten ausgewählt werden.

13. Das Antisense-Oligonukleotid nach Anspruch 10, wobei die modifizierten Phosphate aus Phosphoropiperazidaten ausgewählt werden.

14. Das Antisense-Oligonukleotid nach Anspruch 1, wobei die Zucker-Einheiten des Oligonukleotid-Rückgrats mit unnatürlichen Einheiten ersetzt werden und die Inter-Nukleotid-Bindungen des Oligonukleotid-Rückgrats mit unnatürlichen Inter-Nukleotid-Bindungen ersetzt werden.

15. Das Antisense-Oligonukleotid nach einem der Ansprüche 1-14, wobei die Uracil-Basen Thymin-Basen sind.

16. Das Antisense-Oligonukleotid nach Anspruch 15, wobei das Antisense-Oligonukleotid chemisch an eine oder mehrere Einheiten oder Konjugate gebunden ist, die die Aktivität, die zelluläre Verteilung oder die zelluläre Aufnahme des Antisense-Oligonukleotids verstärken.

17. Das Antisense-Oligonukleotid nach Anspruch 16, wobei das Antisense-Oligonukleotid chemisch an eine Polyethylenglykol-Kette gebunden ist.

18. Eine Zusammensetzung, die ein Antisense-Oligonukleotid gemäß einem der vorangegangenen Ansprüche und einen oder mehrere pharmazeutisch geeignete Träger und/oder Verdünnungsmittel umfasst.

19. Ein Antisense-Oligonukleotid gemäß einem der Ansprüche 1 bis 17 oder eine Zusammensetzung gemäß Anspruch 18 für die Verwendung in einem Verfahren zur Behandlung von muskulärer Dystrophie bei einem Patienten.

Revendications

1. Oligonucléotide antisens qui se lie au gène de dystrophine humaine pour induire un saut d'exon dans le gène de dystrophine, consistant en la séquence de SEQ ID NO : 181, éventuellement où les bases de type uracile (U) sont des bases thymines (T).

2. Oligonucléotide antisens isolé d'une longueur de jusqu'à 50 nucléotides, où l'oligonucléotide antisens comprend l'oligonucléotide antisens selon la revendication 1.

3. Oligonucléotide antisens selon la revendication 1 où l'oligonucléotide antisens comprend un squelette modifié ou des liaisons internucléotidiques non naturelles.

4. Oligonucléotide antisens selon la revendication 1 où l'oligonucléotide antisens est lié chimiquement à un ou plusieurs groupements ou conjugués qui augmentent l'activité, la distribution cellulaire ou l'absorption cellulaire de l'oligonucléotide antisens.

5. Oligonucléotide antisens selon la revendication 1 où l'oligonucléotide antisens n'active pas la RNase H.

6. Oligonucléotide antisens selon la revendication 3 où les groupements glucidiques du squelette de l'oligonucléotide sont remplacés par des groupements non naturels.

7. Oligonucléotide antisens selon la revendication 3 où le squelette modifié comprend des morpholinos.

8. Oligonucléotide antisens selon la revendication 1 où les liaisons internucléotidiques du squelette de l'oligonucléotide sont remplacées par des liaisons internucléotidiques non naturelles.

9. Oligonucléotide antisens selon la revendication 8 où les liaisons internucléotidiques non naturelles sont des phosphates modifiés.

10. Oligonucléotide antisens selon la revendication 9 où les phosphates modifiés sont choisis parmi les méthyl phosphonates, les méthyl phosphorothioates, les phosphoromorpholidates, les phosphoropipérazidates et les phosphoramidates.

11. Oligonucléotide antisens selon la revendication 10 où les phosphates modifiés sont choisis parmi les phosphoramidates.

12. Oligonucléotide antisens selon la revendication 10 où les phosphates modifiés sont choisis parmi les phosphoromorpholidates.

13. Oligonucléotide antisens selon la revendication 10 où les phosphates modifiés sont choisis parmi les phosphoropipérazidates.

14. Oligonucléotide antisens selon la revendication 1 où les groupements glucidiques du squelette de l'oligonucléotide sont remplacés par des groupements non naturels et les liaisons internucléotidiques du squelette de l'oligonucléotide sont remplacées par des liaisons internucléotidiques non naturelles.

15. Oligonucléotide antisens selon l'une quelconque des revendications 1-14 où les bases de type uracile sont des bases thymines.

16. Oligonucléotide antisens selon la revendication 15 où l'oligonucléotide antisens est lié chimiquement à un ou plusieurs groupements ou conjugués qui augmentent l'activité, la distribution cellulaire ou l'absorption cellulaire de l'oligonucléotide antisens.

17. Oligonucléotide antisens selon la revendication 16 où l'oligonucléotide antisens est lié chimiquement à une chaîne de polyéthylèneglycol.

18. Composition comprenant un oligonucléotide antisens selon l'une quelconque des revendications précédentes et un ou plusieurs vecteurs et/ou diluants pharmaceutiquement acceptables.

19. Oligonucléotide antisens selon l'une quelconque des revendications 1 à 17 ou composition selon la revendication 18 destiné(e) à être utilisé(e) dans un procédé de traitement de la dystrophie musculaire chez un patient.

FIGURE 1.

bp Acceptor ESE Donor

ucaugcacugagugaccucuuucucgcagGCGCUAGCUGGAGCA/////CCGUGCAGACUGACGgucucau

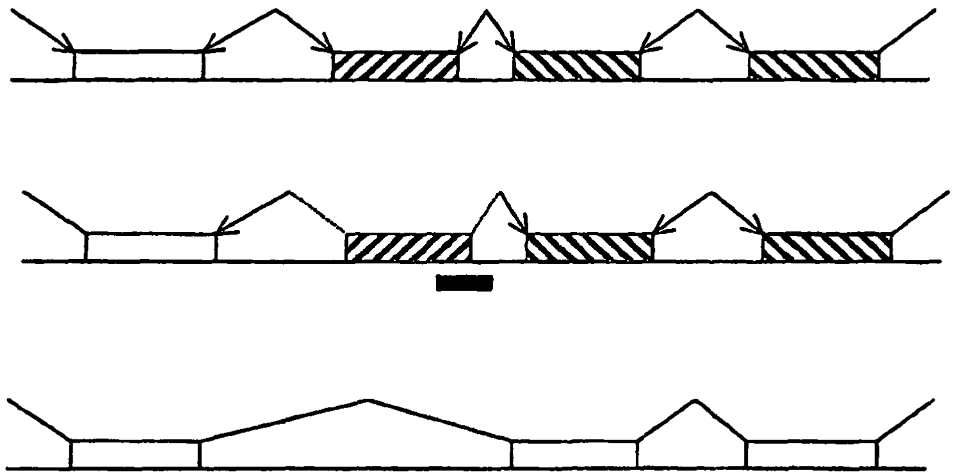


FIGURE 2

REFERENCES CITED IN THE DESCRIPTION

Patent documents cited in the description

- WO 2004083446 A **[0017]**
- US 5149797 A **[0057]**
- US 4458066 A **[0064]**
- US 20040248833 A **[0073]**
- US 6806084 B **[0075]**

Non-patent literature cited in the description

- **Sierakowska H et al.** *Proc Natl Acad Sci USA,* 1996, vol. 93, 12840-12844 **[0005]**
- **Wilton SD et al.** *Neuromusc Disorders,* 1999, vol. 9, 330-338 **[0005]**
- **van Deutekom JC et al.** *Human Mol Genet,* 2001, vol. 10, 1547-1554 **[0005]**
- **Sherrat TG et al.** *Am J Hum Genet,* 1993, vol. 53, 1007-1015 **[0007]**
- **Lu QL et al.** *Nature Medicine,* 2003, vol. 9, 1009-1014 **[0007]**
- **Aartsma-Rus A et al.** *Am J Hum Genet,* 2004, vol. 74, 83-92 **[0007]**
- **Matsuo et al.** *J Clin Invest.,* 1991, vol. 87, 2127-2131 **[0010]**
- **Takeshima et al.** *J. Clin. Invest.,* 1995, vol. 95, 515-520 **[0010]**
- **Dunckley et al.** *Nucleosides & Nucleotides,* 1997, vol. 16, 1665-1668 **[0011]**
- **Dunckley et al.** *Human Mol. Genetics,* 1998, vol. 5, 1083-90 **[0012]**
- **Errington et al.** *J Gen Med,* 2003, vol. 5, 518-527 **[0013]**
- **Wilton et al.** *Neuromuscular Disorders,* 1999, vol. 9, 330-338 **[0015]**
- **Mann CJ et al.** *J Gen Med,* 2002, vol. 4, 644654 **[0016]**
- **Matsuo M.** Duchenne And Becker Muscular Dystrophy: From Gene Diagnosis To Molecular Therapy. *IUBMB LIFE,* 01 March 2002, vol. 53 (3), 147-152 **[0017]**
- **Mann et al.** *J Gen Med,* 2002, vol. 4, 644-654 **[0035]**
- improved antisense oligonucleotide induced exon skipping in the mdx mouse model of muscular dystrophy. *J Gen Med,* vol. 4, 644-654 **[0045]**
- **Beaucage et al.** *Tetrahedron Letters,* 1981, vol. 22, 1859-1862 **[0065]**
- **Martin.** Remington's Pharmaceutical Sciences. Mack Publishing Co, 1990 **[0069]**
- Martin, Remington's Pharmaceutical Sciences. Mack Publishing Co, 1990, 1435-1712 **[0070]**
- **Mann CJ et al.** Antisense-induced exon skipping and the synthesis of dystrophin in the mdx mouse. *Proc., Natl. Acad. Science,* 2001, vol. 98 (1), 42-47 **[0074]**
- **Gebski et al.** *Human Molecular Genetics,* 2003, vol. 12 (15), 1801-1811 **[0074]**
- **Fraley et al.** *Trends Biochem. Sci.,* 1981, vol. 6, 77 **[0077]**
- **Mannino et al.** *Biotechniques,* 1988, vol. 6, 682 **[0078]**
- **Friedmann.** *Science,* 1989, vol. 244, 1275-1280 **[0081]**
- **Rosenberg.** *Cancer Research,* 1991, vol. 51 (18), 5074S-5079S **[0082]**
- **Rosenfeld et al.** *Cell,* 1992, vol. 68, 143-155 **[0082]**
- **Rosenfeld et al.** *Science,* 1991, vol. 252, 431-434 **[0082]**
- **Brigham et al.** *Am. J. Med. Sci.,* 1989, vol. 298, 278-281 **[0082]**
- **Nabel et al.** *Science,* 1990, vol. 249, 1285-1288 **[0082]**
- **Hazinski et al.** *Am. J. Resp. Cell Molec. Biol.,* 1991, vol. 4, 206-209 **[0082]**
- **Wang ; Huang.** *Proc. Natl. Acad. Sci.,* 1987, vol. 84, 7851-7855 **[0082]**
- **Wu ; Wu.** *J. Biol. Chem.,* 1988, vol. 263, 14621-14624 **[0082]**
- **Wolff et al.** *Science,* 1990, vol. 247, 1465-1468 **[0082]**
- **The Brigham et al.** *Am. J. Med. Sci.,* 1989, vol. 298, 278-281 **[0082]**
- *Clinical Research,* 1991, 39 **[0082]**
- **Anderson.** *Science,* 1992, vol. 256, 808-813 **[0082]**
- **Sambrook et al.** Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, 1989 **[0091]**
- DNA Cloning: A Practical Approach. MRL Press, Ltd, 1985, vol. I, II **[0091]**
- **Ausubel, F. ; Brent, R. ; Kingston, R.E. ; Moore, D.D. ; Seidman, J.G. ; Smith, J.A. ; Struhl, K.** Current Protocols In Molecular Biology. Greene Publishing Associates/Wiley Intersciences, 2002 **[0091]**

EXHIBIT AT

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 2 206 781 B1

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
02.12.2015 Bulletin 2015/49

(21) Application number: **10004274.6**

(22) Date of filing: **28.06.2005**

(51) Int Cl.:
C12N 15/113 (2010.01)
A61K 48/00 (2006.01)
A61K 31/7088 (2006.01)
A61K 31/712 (2006.01)
C07H 21/00 (2006.01)

(54) **Antisense oligonucleotides for inducing exon skipping and methods of use thereof**

Antisense-Oligonukleotide zur Induktion von Exon-Skipping sowie Verfahren zur Verwendung davon

Oligonucléotides antisens permettant d'induire un saut d'exon et leurs procédés d'utilisation

(84) Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IS IT LI LT LU MC NL PL PT RO SE SI SK TR
Designated Extension States:
HR LV

(30) Priority: **28.06.2004 AU 2004903474**

(43) Date of publication of application:
14.07.2010 Bulletin 2010/28

(60) Divisional application:
12162995.0 / 2 500 430
15168694.6 / 2 933 332
15190341.6

(62) Document number(s) of the earlier application(s) in accordance with Art. 76 EPC:
05754344.9 / 1 766 010

(73) Proprietor: **THE UNIVERSITY OF WESTERN AUSTRALIA**
Crawley, Western Australia 6009 (AU)

(72) Inventors:
- **Wilton, Stephen Donald**
  Applecross,
  Western Australia 6153 (AU)
- **Fletcher, Sue**
  Bayswater,
  Western Australia 6053 (AU)
- **McClorey, Graham**
  Bayswater,
  Western Australia 6053 (AU)

(74) Representative: **Brasnett, Adrian Hugh et al**
Mewburn Ellis LLP
City Tower
40 Basinghall Street
London EC2V 5DE (GB)

(56) References cited:
WO-A1-93/20227
WO-A2-03/053341
WO-A2-2004/083446
CA-A1- 2 507 125

- **AARTSMA-RUS, A. ET AL.: "Therapeutic antisense-induced exon skipping in cultured muscle cells from six different DMD patients", HUMAN MOLECULAR GENETICS, vol. 12, no. 8, 2003, pages 907-914, XP008084159, ISSN: 0964-6906**
- **AARTSMA-RUS A ET AL: "Targeted exon skipping as a potential gene correction therapy for Duchenne muscular dystrophy", NEUROMUSCULAR DISORDERS,, vol. 12, 1 January 2002 (2002-01-01), pages 71-77, XP008116183, DOI: 10.1016/S0960-8966(02) 00086-X**
- **MATSUO M: "DUCHENNE AND BECKER MUSCULAR DYSTROPHY: FROM GENE DIAGNOSIS TO MOLECULAR THERAPY", IUBMB LIFE, vol. 53, no. 3, 1 March 2002 (2002-03-01), pages 147-152, XP009021242, ISSN: 1521-6543**
- **ERRINGTON STEPHEN J ET AL: "Target selection for antisense oligonucleotide induced exon skipping in the dystrophin gene.", THE JOURNAL OF GENE MEDICINE, vol. 5, no. 6, June 2003 (2003-06), pages 518-527, XP002559309, ISSN: 1099-498X**

Remarks:

•This application was filed on 22-04-2010 as a divisional application to the application mentioned under INID code 62.

•The file contains technical information submitted after the application was filed and not included in this specification

Description

Field of the Invention

[0001] The present invention relates to novel antisense compounds and compositions suitable for facilitating exon skipping. It also provides methods for inducing exon skipping using the novel antisense compounds as well as therapeutic compositions adapted for use in the methods of the invention.

Background Art

[0002] Significant effort is currently being expended researching methods for suppressing or compensating for disease-causing mutations in genes. Antisense technologies are being developed using a range of chemistries to affect gene expression at a variety of different levels (transcription, splicing, stability, translation). Much of that research has focused on the use of antisense compounds to correct or compensate for abnormal or disease-associated genes in a myriad of different conditions.

[0003] Antisense molecules are able to inhibit gene expression with exquisite specificity and because of this many research efforts concerting oligonucleotides as modulators of gene expression have focused on inhibiting the expression of targeted genes such as oncogenes or viral genes. The antisense oligonucleotides are directed either against RNA (sense strand) or against DNA where they form triplex structures inhibiting transcription by RNA polymerase II. To achieve a desired effect in specific gene down-regulation, the oligonucleotides must either promote the decay of the targeted mRNA or block translation of that mRNA, thereby effectively preventing *de novo* synthesis of the undesirable target protein.

[0004] Such techniques are not useful where the object is to up-regulate production of the native protein or compensate for mutations which induce premature termination of translation such as nonsense or frame-shifting mutations. Furthermore, in cases where a normally functional protein is prematurely terminated because of mutations therein, a means for restoring some functional protein production through antisense technology has been shown to be possible through intervention during the splicing processes (Sierakowska H, et al., (1996) Proc Natl Acad Sci USA 93, 12840-12844; Wilton SD, et al., (1999) Neuromusc Disorders 9, 330-338; van Deutekom JC et al., (2001) Human Mol Genet 10, 1547-1554). In these cases, the defective gene transcript should not be subjected to targeted degradation so the antisense oligonucleotide chemistry should not promote target mRNA decay.

[0005] In a variety of genetic diseases, the effects of mutations on the eventual expression of a gene can be modulated through a process of targeted exon skipping during the splicing process. The splicing process is directed by complex multi-particle machinery that brings adjacent exon-intron junctions in pre-mRNA into close proximity and performs cleavage of phosphodiester bonds at the ends of the introns with their subsequent reformation between exons that are to be spliced together. This complex and highly precise process is mediated by sequence motifs in the pre-mRNA that are relatively short semi-conserved RNA segments to which bind the various nuclear splicing factors that are then involved in the splicing reactions. By changing the way the splicing machinery reads or recognises the motifs involved in pre-mRNA processing, it is possible to create differentially spliced mRNA molecules. It has now been recognised that the majority of human genes are alternatively spliced during normal gene expression, although the mechanism invoked have not been identified. Using antisense oligonucleotides, it has been shown that errors and deficiencies in a coded mRNA could be bypassed or removed from the mature gene transcripts.

[0006] In nature, the extent of genetic deletion or exon skipping in the splicing process is not fully understood, although many instances have been documented to occur, generally at very low levels (Sherrat TG, et al., (1993) Am J Hum Genet 53, 1007-1015). However, it is recognised that if exons associated with disease-causing mutations can be specifically deleted from some genes, a shortened protein product can sometimes be produced that has similar biological properties of the native protein or has sufficient biological activity to ameliorate the disease caused by mutations associated with the target exon (Lu QL, et al., (2003) Nature Medicine 9, 1009-1014; Aartsma-Rus A et al., (2004) Am J Hum Genet 74: 83-92).

[0007] This process of targeted exon skipping is likely to be particularly useful in long genes where there are many exons and introns, where there is redundancy in the genetic constitution of the exons or where a protein is able to function without one or more particular exons (e.g. with the dystrophin gene, which consists of 79 exons; or possibly some collagen genes which encode for repeated blocks of sequence or the huge nebulin or titin genes which are comprised of ~80 and over 370 exons, respectively).

[0008] Efforts to redirect gene processing for the treatment of genetic diseases associated with truncations caused by mutations in various genes have focused on the use of antisense oligonucleotides that either (1) fully or partially overlap with the elements involved in the splicing process; or (2) bind to the pre-mRNA at a position sufficiently close to the element to disrupt the binding and function of the splicing factors that would normally mediate a particular splicing reaction which occurs at that element (e.g., binds to the pre-mRNA at a position within 3, 6, or 9 nucleotides of the

element to be blocked).

[0009] For example, modulation of mutant dystrophin pre-mRNA splicing with antisense oligoribonucleotides has been reported both *in vitro* and *in vivo*. In one type of dystrophin mutation reported in Japan, a 52-base pair deletion mutation causes exon 19 to be removed with the flanking introns during the splicing process (Matsuo et al., (1991) J Clin Invest. 87:2127-2131). An *in vitro* minigene splicing system has been used to show that a 31-mer 2'-O-methyl oligoribonucleotide complementary to the 5' half of the deleted sequence in dystrophin Kobe exon 19 inhibited splicing of wild-type *pre-mRNA* (Takeshima et al. (1995), J. Clin. Invest., 95, 515-520). The same oligonucleotide was used to induce exon skipping from the native dystrophin gene transcript in human cultured lymphoblastoid cells.

[0010] Dunckley et al., (1997) Nucleosides & Nucleotides, 16, 1665-1668 described *in vitro* constructs for analysis of splicing around exon 23 of mutated dystrophin in the *mdx* mouse mutant, a model for muscular dystrophy. Plans to analyse these constructs *in vitro* using 2' modified oligonucleotides targeted to splice sites within and adjacent to mouse dystrophin exon 23 were discussed, though no target sites or sequences were given.

[0011] 2'-O-methyl oligoribonucleotides were subsequently reported to correct dystrophin deficiency in myoblasts from the *mdx* mouse from this group. An antisense oligonucleotide targeted to the 3' splice site of murine dystrophin intron 22 was reported to cause skipping of the mutant exon as well as several flanking exons and created a novel in-frame dystrophin transcript with a novel internal deletion. This mutated dystrophin was expressed in 1-2% of antisense treated mdx myotubes. Use of other oligonucleotide modifications such as 2'-O-methoxyethyl phosphodiesters are described (Dunckley et al. (1998) Human Mol. Genetics, 5, 1083-90).

[0012] Thus, antisense molecules may provide a tool in the treatment of genetic disorders such as Duchenne Muscular Dystrophy (DMD). However, attempts to induce exon skipping using antisense molecules have had mixed success. Studies on dystrophin exon 19, where successful skipping of that exon from the dystrophin pre-mRNA was achieved using a variety of antisense molecules directed at the flanking splice sites or motifs within the exon involved in exon definition as described by Errington et al. (2003) J Gen Med 5, 518-527".

[0013] In contrast to the apparent ease of exon 19 skipping, the first report of exon 23 skipping in the *mdx* mouse by Dunckley *et al*., (1998) is now considered to be reporting only a naturally occurring revertant transcript or artefact rather than any true antisense activity. In addition to not consistently generating transcripts missing exon 23, Durickley *et al*., (1998) did not show any time course of induced exon skipping, or even titration of antisense oligonucleotides, to demonstrate dose dependent effects where the levels of exon skipping corresponded with increasing or decreasing amounts of antisense oligonucleotide. Furthermore, this work could not be replicated by other researchers.

[0014] The first example of specific and reproducible exon skipping in the *mdx* mouse model was reported by Wilton et al., (1999) Neuromuscular Disorders 9, 330-338. By directing an antisense molecule to the donor splice site, consistent and efficient exon 23 skipping was induced in the dystrophin mRNA within 6 hours of treatment of the cultured cells. Wilton *et al*., (1999), also describe targeting the acceptor-region of the mouse dystrophin pre-mRNA with longer antisense oligonucleotides and being unable to repeat the published results of Dunckley *et al*., (1998). No exon skipping, either 23 alone or multiple removal of several flanking exons, could be reproducibly detected using a selection of antisense oligonucleotides directed at the acceptor splice site of intron 22.

[0015] While the first antisense oligonucleotide directed at the intron 23 donor splice site induced consistent exon skipping in primary cultured myoblasts, this compound was found to be much less efficient in immortalized cell cultures expressing higher levels of dystrophin. However, with refined targeting and antisense oligonucleotide design, the efficiency of specific exon removal was increased by almost an order of magnitude (see Mann CJ et al., (2002) J Gen Med 4, 644-654).

[0016] Other disclosures relating to DMD therapy include CA 2507125, Aartsma-Rus et al., Human Molecular Genetics 12, (2003) 907-14, Aartsma-rus et al., Neuromuscular Disorders, 12 (2002) 71-7, as well as WO 2004/083446, published on 30 September 2004.

[0017] Thus, there remains a need to provide antisense oligonucleotides capable of binding to and modifying the splicing of a target nucleotide sequence. Simply directing the antisense oligonucleotides to motifs presumed to be crucial for splicing is no guarantee of the efficacy of that compound in a therapeutic setting.

Summary of the Invention

[0018] The present invention provides an isolated antisense oligonucleotide that binds to human dystrophin pre-mRNA, wherein said oligonucleotide is 20 to 31 nucleotides in length and is an oligonucleotide that is specifically hybridizable to an exon 53 target region of the Dystrophin gene designated as annealing site H53A (+23+47), annealing site H53A (+39+69), or both, wherein said antisense oligonucleotide is a morpholino antisense oligonucleotide, and, wherein said oligonucleotide induces exon 53 skipping..

[0019] The invention further provides a composition comprising an antisense oligonucleotide according to the invention and a saline solution that includes a phosphate buffer.

[0020] The invention further provides an antisense oligonucleotide according to the invention, or a composition ac-

cording to the invention, for use in a method of treatment of muscular dystrophy.

[0021] The invention is further defined in the accompanying claims.

[0022] The choice of target selection plays a crucial role in the efficiency of exon skipping and hence its subsequent application of a potential therapy. Simply designing antisense molecules to target regions of pre-mRNA presumed to be involved in splicing is no guarantee of inducing efficient and specific exon skipping. The most obvious or readily defined targets for splicing intervention are the donor and acceptor splice sites although there are less defined or conserved motifs including exonic splicing enhancers, silencing elements and branch points.

[0023] The acceptor and donor splice sites have consensus sequences of about 16 and 8 bases respectively (see Figure 1 for schematic representation of motifs and domains involved in exon recognition, intron removal and the splicing process).

[0024] The invention also addresses the use of purified and isolated antisense oligonucleotides of the invention, for the manufacture of a medicament for treatment of a genetic disease.

[0025] The invention may be used for treating a condition characterised by Duchenne muscular dystrophy, by administering to a patient in need of treatment an effective amount of an appropriately designed antisense oligonucleotide of the Invention, relevant to the particular genetic lesion in that patient.

[0026] Further, the invention may be used for prophylactically treating a patient to prevent or at least minimize Duchenne muscular dystrophy, comprising the step of: administering to the patient an effective amount of an antisense oligonucleotide or a pharmaceutical composition comprising one or more of these biological molecules.

[0027] Also described herein are kits for treating a genetic disease, which kits comprise at least a antisense oligonucleotide of the present invention, packaged in a suitable container and instructions for its use.

[0028] Other aspects and advantages of the invention will become apparent to those skilled in the art from a review of the ensuing description, which proceeds with reference to the following figures.

Brief Description of the Drawings

[0029]

Figure 1 Schematic representation of motifs and domains Involved in exon recognition, intron removal and the splicing process.

Figure 2 Diagrammatic representation of the concept of antisense oligonucleotide induced exon skipping to by-pass disease-causing mutations (not drawn to scale). The hatched box represents an exon carrying a mutation that prevents the translation of the rest of the mRNA into a protein. The solid black bar represents an antisense oligonucleotide that prevents inclusion of that exon in the mature mRNA.

Figure 3 Gel electrophoresis showing exon 51, exon 52 and exon 53 skipping using various antisense molecules directed at exons 51, 52 and 53, respectively. A "cocktail" of antisense molecules is also shown directed at exon 53

Brief Description of the Sequence Listings

[0030]

Table 1: Description of 2'-O-methyl phosphorothioate antisense oligonucleotides that have been used to date to study induced exon skipping during the processing of the dystrophie pre-mRNA. Since these 2'-O-methyl antisense oligonucleotides are more RNA-like, U represents uracil. With other antisense chemistries such as peptide nucleic acids or morpholinos, these U bases may be shown as "**T**".

SEQ ID	SEQUENCE	NUCLEOTIDE SEQUENCE (5'-3')
192	H53A(+39+62)	CUG UUG CCU CCG GUU CUG AAG GUG
193	H53A(+39+69)	$\boxed{\text{CAU UCA ACU GUU GCC UCC GGU UCU GAA GGU G}}$
195	H53A(+23+47)	CUG AAG GUG UUC UUG UAC UUC AUC C

Detailed Description of the Invention

General

[0031] Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variation and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in the specification, individually or collectively and any and all combinations or any two or more of the steps or features.

[0032] The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purpose of exemplification only. Functionally equivalent products, compositions and methods are clearly within the scope of the invention as described herein.

[0033] Sequence identity numbers (SEQ ID NO:) containing nucleotide and amino acid sequence information included in this specification are collected at the end of the description and have been prepared using the programme PatentIn Version 3.0. Each nucleotide or amino acid sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier (e.g. <210>1, <210>2, etc.). The length, type of sequence and source organism for each nucleotide or amino acid sequence are indicated by information provided in the numeric indicator fields <211>, <212> and <213>, respectively. Nucleotide and amino acid sequences referred to in the specification are defined by the information provided in numeric indicator field <400> followed by the sequence identifier (e.g. <400>1, <400>2, etc.).

[0034] An antisense molecules nomenclature system was proposed and published to distinguish between the different antisense molecules (see Mann et al., (2002) J Gen Med 4, 644-654). This nomenclature became especially relevant when testing several slightly different antisense molecules, all directed at the same target region, as shown below:

H # A/D (x : y).

[0035] The first letter designates the species (e.g. H: human, M: murine, C: canine)

[0036] "#" designates target dystrophin exon number.

[0037] "A/D" indicates acceptor or donor splice site at the beginning and end of the exon, respectively.

[0038] (x y) represents the annealing coordinates where "-" or "+" indicate intronic or exonic sequences respectively. As an example, A(-6+18) would indicate the last 6 bases of the intron preceding the target exon and the first 18 bases of the target exon. The closest splice site would be the acceptor so these coordinates would be preceded with an "A". Describing annealing coordinates at the donor splice site could be D(+2-18) where the last 2 exonic bases and the first 18 intronic bases correspond to the annealing site of the antisense molecule. Entirely exonic annealing coordinates that would be represented by A(+65+85), that is the site between the 65th and 85th nucleotide from the start of that exon.

[0039] No admission is made that any of the references constitute prior art or are part of the common general knowledge of those working in the field to which this invention relates.

[0040] As used necessarily herein the term "derived" and "derived from" shall be taken to indicate that a specific integer may be obtained from a particular source *albeit* not directly from that source.

[0041] Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

[0042] Other definitions for selected terms used herein may be found within the detailed description of the invention and apply throughout. Unless otherwise defined, all other scientific and technical terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which the invention belongs.

Description of the Preferred Embodiment

[0043] When antisense molecule(s) are targeted to nucleotide sequences involved in splicing in exons within pre-mRNA sequences, normal splicing of the exon may be inhibited causing the splicing machinery to by-pass the entire mutated exons from the mature mRNA. The concept of antisense oligonucleotide induced exon skipping is shown in Figure 2. In many genes, deletion of an entire exon would lead to the production of a non-functional protein through the loss of important functional domains or the disruption of the reading frame. In some proteins, however, it is possible to shorten the protein by deleting one or more exons, without disrupting the reading frame, from within the protein without seriously altering the biological activity of the protein. Typically, such proteins have a structural role and or possess functional domains at their ends. The present invention describes antisense molecules capable of binding to specified dystrophin pre-mRNA targets and re-directing processing of that gene.

Antisense Molecules

[0044] According to a first aspect of the invention, there is provided antisense molecules as defined in the claims capable of binding to a selected target to induce exon skipping. To induce exon skipping in exons of the Dystrophin gene transcript, the antisense molecules are preferably selected from the group of compounds shown in Table 1. Also described is a combination or "cocktail" of two or more antisense oligonucleotides capable of binding to a selected target to induce exon skipping.

[0045] Designing antisense molecules to completely mask consensus splice sites may not necessarily generate any skipping of the targeted exon. Furthermore, the inventors have discovered that size or length of the antisense oligonucleotide itself is not always a primary factor when designing antisense molecules. With some targets such as exon 19, antisense oligonucleotides as short as 12 bases were able to induce exon skipping, albeit not as efficiently as longer (20-31 bases) oligonucleotides. In some other targets, such as murine dystrophin exon 23, antisense oligonucleotides only 17 residues long were able to induce more efficient skipping than another overlapping compound of 25 nucleotides.

[0046] The inventors have also discovered that there does not appear to be any standard motif that can be blocked or masked by antisense molecules to redirect splicing. In some exons, such as mouse dystrophin exon 23, the donor splice site was the most amenable to target to re-direct skipping of that exon. It should be noted that designing and testing a series of exon 23 specific antisense molecules to anneal to overlapping regions of the donor splice site showed considerable variation in the efficacy of induced exon skipping. As reported in Mann et al., (2002) there was a significant variation in the efficiency of bypassing the nonsense mutation depending upon antisense oligonucleotide annealing ("Improved antisense oligonucleotide induced exon skipping in the mdx mouse model of muscular dystrophy". J Gen Med 4: 644-654). Targeting the acceptor site of exon 23 or several internal domains was not found to induce any consistent exon 23 skipping.

[0047] In other exons targeted for removal, masking the donor splice site did not induce any exon skipping. However, by directing antisense molecules to the acceptor splice site (human exon 8), strong and sustained exon skipping was induced. It should be noted that removal of human exon 8 was tightly linked with the co-removal of exon 9. There is no strong sequence homology between the exon 8 antisense oligonucleotides and corresponding regions of exon 9 so it does not appear to be a matter of cross reaction. Rather the splicing of these two exons is inextricably linked. This is not an isolated instance as the same effect is observed in canine cells where targeting exon 8 for removal also resulted in the skipping of exon 9. Targeting exon 23 for removal in the mouse dystrophin pre-mRNA also results in the frequent removal of exon 22 as well. This effect occurs in a dose dependent manner and also indicates close coordinated processing of 2 adjacent exons.

[0048] In other targeted exons, antisense molecules directed at the donor or acceptor splice sites did not induce exon skipping while annealing antisense molecules to intra-exonic regions (i.e. exon splicing enhancers within human dystrophin exon 6) was most efficient at inducing exon skipping. Some exons, both mouse and human exon 19 for example, are readily skipped by targeting antisense molecules to a variety of motifs. That is, targeted exon skipping is induced after using antisense oligonucleotides to mask donor and acceptor splice sites or exon splicing enhancers.

[0049] To identify and select antisense oligonucleotides suitable for use in the modulation of exon skipping, a nucleic acid sequence whose function is to be modulated must first be identified. This may be, for example, a gene (or mRNA transcribed form the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. Within the context of the present invention, preferred target site(s) are those involved in mRNA splicing (i.e. splice donor sites, splice acceptor sites, or exonic splicing enhancer elements). Splicing branch points and exon recognition sequences or splice enhancers are also potential target sites for modulation of mRNA splicing.

[0050] Preferably, the present invention aims to provide antisense molecules capable of binding to a selected target in the dystrophin pre-mRNA to induce efficient and consistent exon skipping. Duchenne muscular dystrophy arises from mutations that preclude the synthesis of a functional dystrophin gene product. These Duchenne muscular dystrophy gene defects are typically nonsense mutations or genomic rearrangements such as deletions, duplications or microdeletions or insertions that disrupt the reading frame. As the human dystrophin gene is a large and complex gene with the 79 exons being spliced together to generate a mature mRNA with an open reading frame of approximately 11,000 bases, there are many positions where these mutations can occur. Consequently, a comprehensive antisense oligonucleotide based therapy to address many of the different disease-causing mutations in the dystrophin gene will require that many exons can be targeted for removal during the splicing process.

[0051] Within the context of the present invention, preferred target site(s) are those involved in mRNA splicing (i.e. splice donor sites, splice acceptor sites or exonic splicing enhancer elements). Splicing branch points and exon recognition sequences or splice enhancers are also potential target sites for modulation of mRNA splicing.

[0052] The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridisable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA

target.

[0053] It is understood in the art that the sequence of an antisense molecule need not be 100% complementary to that of its target sequence to be specifically hybridisable. An antisense molecule is specifically hybridisable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment, and in the case of *in vitro* assays, under conditions in which the assays are performed.

[0054] While the above method may be used to select antisense molecules capable of deleting any exon from within a protein that is capable of being shortened without affecting its biological function, the exon deletion should not lead to a reading frame shift in the shortened transcribed mRNA. Thus, if in a linear sequence of three exons the end of the first exon encodes two of three nucleotides in a codon and the next exon is deleted then the third exon in the linear sequence must start with a single nucleotide that is capable of completing the nucleotide triplet for a codon. If the third exon does not commence with a single nucleotide there will be a reading frame shift that would lead to the generation of truncated or a non-functional protein.

[0055] It will be appreciated that the codon arrangements at the end of exons in structural proteins may not always break at the end of a codon, consequently there may be a need to delete more than one exon from the pre-mRNA to ensure in-frame reading of the mRNA. In such circumstances, a plurality of antisense oligonucleotides may need to be selected by the method of the invention wherein each is directed to a different region responsible for inducing splicing in the exons that are to be deleted.

[0056] The length of an antisense molecule may vary so long as it is capable of binding selectively to the intended location within the pre-mRNA molecule. The length of such sequences can be determined in accordance with selection procedures described herein. Generally, the antisense molecule will be from about 10 nucleotides in length up to about 50 nucleotides in length. It will be appreciated however that any length of nucleotides within this range may be used in the method. Preferably, the length of the antisense molecule is between 17 to 30 nucleotides in length.

[0057] In order to determine which exons can be connected in a dystrophin gene, reference should be made to an exon boundary map. Connection of one exon with another is based on the exons possessing the same number at the 3' border as is present at the 5' border of the exon to which it is being connected. Therefore, if exon 7 were deleted, exon 6 must connect to either exons 12 or 18 to maintain the reading frame. Thus, antisense oligonucleotides would need to be selected which redirected splicing for exons 7 to 11 in the first instance or exons 7 to 17 in the second instance. Another and somewhat simpler approach to restore the reading frame around an exon 7 deletion would be to remove the two flanking exons. Induction of exons 6 and 8 skipping should result in an in-frame transcript with the splicing of exons 5 to 9. In practise however, targeting exon 8 for removal from the pre-mRNA results in the co-removal of exon 9 so the resultant transcript would have exon 5 joined to exon 10. The inclusion or exclusion of exon 9 does not alter the reading frame. Once the antisense molecules to be tested have been identified, they are prepared according to standard techniques known in the art. The most common method for producing antisense molecules is the methylation of the 2' hydroxyribose position and the incorporation of a phosphorothioate backbone produces molecules that superficially resemble RNA but that are much more resistant to nuclease degradation.

[0058] To avoid degradation of pre-mRNA during duplex formation with the antisense molecules, the antisense molecules used in the method may be adapted to minimise or prevent cleavage by endogenous RNase H. This property is highly preferred as the treatment of the RNA with the unmethylated oligonucleotides either intracellularly or in crude extracts that contain RNase H leads to degradation of the pre-mRNA: antisense oligonucleotide duplexes. Any form of modified antisense molecules that is capable of by-passing or not inducing such degradation may be used in the present method. An example, of antisense molecules which when duplexed with RNA are not cleaved by cellular RNase H is 2'-O-methyl derivatives. 2'-O-methyl-oligoribonucleotides are very stable in a cellular environment and in animal tissues, and their duplexes with RNA have higher Tm values than their ribo- or deoxyribo- counterparts.

[0059] Antisense molecules that do not activate RNase H can be made in accordance with known techniques (see, e.g., U.S. Pat. 5,149,797). Such antisense molecules, which may be deoxyribonucleotide or ribonucleotide sequences, simply contain any structural modification which sterically hinders or prevents binding of RNase H to a duplex molecule containing the oligonucleotide as one member thereof, which structural modification does not substantially hinder or disrupt duplex formation. Because the portions of the oligonucleotide involved in duplex formation are substantially different from those portions involved in RNase H binding thereto, numerous antisense molecules that do not activate RNase H are available. For example, such antisense molecules may be oligonucleotides wherein at least one, or all, of the inter-nucleotide bridging phosphate residues are modified phosphates, such as methyl phosphonates, methyl phosphorothioates, phosphoromorpholidates, phosphoropiperazidates and phosphoramidates. For example, every other one of the internucleotide bridging phosphate residues may be modified as described. In another nonlimiting example, such antisense molecules are molecules wherein at least one, or all, of the nucleotides contain a 2' lower alkyl moiety (e.g., C_1-C_4, linear or branched, saturated or unsaturated alkyl, such as methyl, ethyl, ethenyl, propyl, 1-propenyl, 2-propenyl,

and isopropyl). For example, every other one of the nucleotides may be modified as described.

[0060] While antisense oligonucleotides are a preferred form of the antisense molecules, the present invention comprehends other oligomeric antisense molecules, including but not limited to oligonucleotide mimetics such as are described below.

[0061] Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural inter-nucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom In their inter-nucleoside backbone can also be considered to be oligonucleosides.

[0062] In other preferred oligonucleotide mimetics, both the sugar and the inter-nucleoside linkage, i.e., the backbone; of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleo-bases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone.

[0063] Modified oligonucleotides may also contain one or more substituted sugar moieties. Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. Certain nucleo-bases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These Include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

[0064] Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates that enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety, cholic acid, a thioether, e.g., hexyl-S-tritylthiol, a thiocholesterol, an aliphatic chain, e.g., dodecandiol or undecyl residues, a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate, a polyamine or a polyethylene glycol chain, or adamantane acetic acid, a palmityl moiety, or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety.

[0065] It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes antisense compounds that are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense molecules, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the Increased resistance to nuclease degradation, increased cellular uptake, and an additional region for increased binding affinity for the target nucleic acid.

Methods of Manufacturing Antisense Molecules

[0066] The antisense molecules used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, Calif.). One method for synthesising oligonucleotides on a modified solid support is described in U.S. Pat. No. 4,458,066.

[0067] Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives. In one such automated embodiment, diethyl-phosphoramidites are used as starting materials and may be synthesized as described by Beaucage, et al., (1981) Tetrahedron Letters, 22:1859-1862.

[0068] The antisense molecules of the invention are synthesised *in vitro* and do not include antisense compositions of biological origin, or genetic vector constructs designed to direct the *in vivo* synthesis of antisense molecules. The molecules of the invention may also be mixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption.

Therapeutic Agents

[0069] The present invention also can be used as a prophylactic or therapeutic, which may be utilised for the purpose

of treatment of a genetic disease.

[0070] Accordingly, in one embodiment the present invention provides antisense molecules that bind to a selected target in the dystrophin pre-mRNA to induce efficient and consistent exon skipping described herein in a therapeutically effective amount admixed with a pharmaceutically acceptable carrier, diluent, or excipient.

[0071] The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similarly untoward reaction, such as gastric upset and the like, when administered to a patient. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solution. Suitable pharmaceutical carriers are described in Martin, Remington's Pharmaceutical Sciences, 18th Ed., Mack Publishing Co., Easton, PA, (1990).

[0072] In a more specific form of the invention there are provided pharmaceutical compositions comprising therapeutically effective amounts of an antisense molecule together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content (*e.g.*, Tris-HCl, acetate, phosphate), pH and ionic strength and additives such as detergents and solubilizing agents (e.g., Tween 80, Polysorbate 80), anti-oxidants (*e.g.*, ascorbic acid, sodium metabisulfite), preservatives (*e.g.,* Thimersol, benzyl alcohol) and bulking substances (*e.g.,* lactose, mannitol). The material may be incorporated into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into liposomes. Hylauronic acid may also be used. Such compositions may influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the present proteins and derivatives. *See, e.g.,* Martin, Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435-1712. The compositions may be prepared in liquid form, or may be in dried powder, such as lyophilised form.

[0073] It will be appreciated that pharmaceutical compositions provided according to the present invention may be administered by any means known in the art. Preferably, the pharmaceutical compositions for administration are administered by injection, orally, or by the pulmonary, or nasal route. The antisense molecules are more preferably delivered by intravenous, intra-arterial, intraperitoneal, intramuscular, or subcutaneous routes of administration.

<u>Antisense molecule based therapy</u>

[0074] Also addressed by the present invention is the use of antisense molecules of the present invention, for manufacture of a medicament for modulation of a genetic disease.

[0075] The delivery of a therapeutically useful amount of antisense molecules may be achieved by methods previously published. For example, intracellular delivery of the antisense molecule may be via a composition comprising an admixture of the antisense molecule and an effective amount of a block copolymer. An example of this method is described in US patent application US 20040248833.

[0076] Other methods of delivery of antisense molecules to the nucleus are described in Mann CJ et al., (2001) ["Antisense-induced exon skipping and the synthesis of dystrophin in the mdx mouse". Proc., Natl. Acad. Science, 98(1) 42-47] and in Gebski et al., (2003). Human Molecular Genetics, 12(15): 1801-1811.

[0077] A method for introducing a nucleic acid molecule into a cell by way of an expression vector either as naked DNA or complexed to lipid carriers, is described in US patent US 6,806,084.

[0078] It may be desirable to deliver the antisense molecule in a colloidal dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes or liposome formulations.

[0079] Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. These formulations may have net cationic, anionic or neutral charge characteristics and are useful characteristics with *in vitro, in vivo* and *ex vivo* delivery methods. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0 .PHI.m can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, and DNA can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, et al., Trends Biochem. Sci., 6:77, 1981).

[0080] In order for a liposome to be an efficient gene transfer vehicle, the following characteristics should be present: (1) encapsulation of the antisense molecule of interest at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino, et al., Biotechniques, 6:682, 1988).

[0081] The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of

divalent cations.

[0082] Alternatively, the antisense construct may be combined with other pharmaceutically acceptable carriers or diluents to produce a pharmaceutical composition. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular, oral or transdermal administration.

[0083] The routes of administration described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and any dosage for any particular animal and condition. Multiple approaches for introducing functional new genetic material into cells, both in vitro and in vivo have been attempted (Friedmann (1989) Science, 244:1275-1280).

[0084] These approaches include integration of the gene to be expressed into modified retroviruses (Friedmann (1989) supra; Rosenberg (1991) Cancer Research 51(18), suppl.: 5074S-5079S); integration into non-retrovirus vectors (Rosenfeld, et al. (1992) Cell, 68:143-155; Rosenfeld, et al. (1991) Science, 252:431-434); or delivery of a transgene linked to a heterologous promoter-enhancer element via liposomes (Friedmann (1989), supra; Brigham, et al. (1989) Am. J. Med. Sci.. 298:278-281; Nabel, et al. (1990) Science, 249:1285-1288; Hazinski, et al. (1991) Am. J. Resp. Cell Molec. Biol., 4:206-209; and Wang and Huang (1987) Proc. Natl. Acad. Sci. (USA), 84:7851-7855); coupled to ligand-specific, cation-based transport systems (Wu and Wu (1988) J. Biol. Chem., 263:14621-14624) or the use of naked DNA, expression vectors (Nabel et al. (1990), supra); wolff et al. (1990) Science, 247:1465-1468). Direct injection of transgenes into tissue produces only localized expression (Rosenfeld (1992) supra); Rosenfeld et al. (1991) supra; Brigham et al. (1989) supra; Nabel (1990) supra; and Hazinski et al. (1991) supra). The Brigham et al. group (Am. J. Med. Sci. (1989) 298:278-281 and Clinical Research (1991) 39 (abstract)) have reported in vivo transfection only of lungs of mice following either intravenous or intratracheal administration of a DNA liposome complex. An example of a review article of human gene therapy procedures is: Anderson, Science (1992) 256:808-813.

[0085] The antisense molecules of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such pro-drugs, and other bioequivalents.

[0086] The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

[0087] For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, (including by nebulizer, intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intra-arterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

[0088] The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Kits of the Invention

[0089] The invention also provides kits for treatment of a patient with a genetic disease which kit comprises at least an antisense molecule, packaged in a suitable container, together with instructions for its use.

[0090] In a preferred embodiment, the kits will contain at least one antisense molecule as defined in the claims. The kits may also contain peripheral reagents such as buffers, stabilizers, etc.

[0091] Those of ordinary skill in the field should appreciate that applications of the above method has wide application

for identifying antisense molecules suitable for use in the treatment of many other diseases.

EXAMPLES

[0092] The following Examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these Examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes.

[0093] Methods of molecular cloning, immunology and protein chemistry, which are not explicitly described in the following examples, are reported in the literature and are known by those skilled in the art. General texts that described conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art, included, for example: Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989); Glover ed., DNA Cloning: A Practical Approach, Volumes I and II, MRL Press, Ltd., Oxford, U.K. (1985); and Ausubel, F., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. Current Protocols in Molecular Biology. Greene Publishing Associates/Wiley Intersciences, New York (2002).

Determining Induced Exon Skipping in Human Muscle Cells

[0094] Attempts by the inventors to develop a rational approach in antisense molecules design were not completely successful as there did not appear to be a consistent trend that could be applied to all exons. As such, the identification of the most effective and therefore most therapeutic antisense molecules compounds has been the result of empirical studies.

[0095] These empirical studies involved the use of computer programs to identify motifs potentially involved in the splicing process. Other computer programs were also used to identify regions of the pre-mRNA which may not have had extensive secondary structure and therefore potential sites for annealing of antisense molecules. Neither of these approaches proved completely reliable in designing antisense oligonucleotides for reliable and efficient induction of exon skipping.

[0096] Annealing sites on the human dystrophin pre-mRNA were selected for examination, initially based upon known or predicted motifs or regions involved in splicing. 2OMe antisense oligonucleotides were designed to be complementary to the target sequences under investigation and were synthesised on an Expedite 8909 Nucleic Acid Synthesiser. Upon completion of synthesis, the oligonucleotides were cleaved from the support column and de-protected in ammonium hydroxide before being desalted. The quality of the oligonucleotide synthesis was monitored by the intensity of the trityl signals upon each deprotection step during the synthesis as detected in the synthesis log. The concentration of the antisense oligonucleotide was estimated by measuring the absorbance of a diluted aliquot at 260nm.

[0097] Specified amounts of the antisense molecules were then tested for their ability to induce exon skipping in an *in vitro* assay, as described below.

[0098] Briefly, normal primary myoblast cultures were prepared from human muscle biopsies obtained after informed consent. The cells were propagated and allowed to differentiate into myotubes using standard culturing techniques. The cells were then transfected with the antisense oligonucleotides by delivery of the oligonucleotides to the cells as cationic lipoplexes, mixtures of antisense molecules or cationic liposome preparations.

[0099] The cells were then allowed to grow for another 24 hours, after which total RNA was extracted and molecular analysis commenced. Reverse transcriptase amplification (RT-PCR) was undertaken to study the targeted regions of the dystrophin pre-mRNA or induced exonic re-arrangements.

[0100] For example, in the testing of an antisense molecule for inducing exon 19 skipping the RT-PCR test scanned several exons to detect involvement of any adjacent exons. For example, when inducing skipping of exon 19, RT-PCR was carried out with primers that amplified across exons 17 and 21. Amplifications of even larger products in this area (i.e. exons 13-26) were also carried out to ensure that there was minimal amplification bias for the shorter induced skipped transcript. Shorter or exon skipped products tend to be amplified more efficiently and may bias the estimated of the normal and induced transcript.

[0101] The sizes of the amplification reaction products were estimated on an agarose gel and compared against appropriate size standards. The final confirmation of identity of these products was carried out by direct DNA sequencing to establish that the correct or expected exon junctions have been maintained.

[0102] Once efficient exon skipping had been induced with one antisense molecule, subsequent overlapping antisense molecules may be synthesized and then evaluated in the assay as described above. Our definition of an efficient antisense molecule is one that induces strong and sustained exon skipping at transfection concentrations in the order of 300 nM or less.

Antisense Oligonucleotides Directed at Exon 53

[0103] Antisense oligonucleotides directed at exon 53 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

[0104] Figure 3 also shows antisense molecule H53A(+39+69) [SEQ ID NO:193] directed at exon 53 acceptor splice site. This antisense oligonucleotide was able to induce exon 53 skipping at 5, 100, 300 and 600 nM. A "cocktail" of three exon 53 antisense oligonucleotides:- H53D(+23+47) [SEQ ID NO:195], H53A(+150+175) [SEQ ID NO:196] and H53A(+14-07) [SEQ ID NO:194], were also tested, as shown in Figure 3 and exhibited an ability to induce exon skipping.

[0105] Table 2 below includes other antisense molecules tested at a concentration range of 50, 100, 300 and 600 nM. These antisense molecules showed varying ability to induce exon 53 skipping. Antisense molecule H53A(+39+69) [SEQ ID NO:193] induced the strongest exon 53 skipping.

Table 2

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H53A(+45+69)	CAU UCA ACU GUU GCC UCC GGU UCU G	Faint skipping at 50 nM
H53A(+39+62)	CUG UUG CCU CCG GUU CUG AAG GUG	Faint skipping at 50 nM
H53A(+39+69)	CAU UCA ACU GUU GCC UCC GGU UCU GAA GGU G	Strong skipping to 50 nM
H53D(+14-07)	UAC UAA CCU UGG UUU CUG UGA	Very faint skipping to 50 nM
H53A(+23+47)	CUG AAG GUG UUC UUG UAC UUC AUC C	Very faint skipping to 50 nM
H53A(+150+176)	UGU AUA GGG ACC CUC CUU CCA UGA CUC	Very faint skipping to 50 nM
H53D(+20-05)	CUA ACC UUG GUU UCU GUG AUU UUC U	Not made yet
H53D(+09-18)	GGU AUC UUU GAU ACU AAC CUU GGU UUC	Faint at 600 nM
H53A(-12+10)	AUU CUU UCA ACU AGA AUA AAA G	No skipping
H53A(-07+18)	GAU UCU GAA UUC UUU CAA CUA GAA U	No skipping
H53A(+07+26)	AUC CCA CUG AUU CUG AAU UC	No skipping
H53A(+124+145)	UUG GCU CUG GCC UGU CCU AAG A	No skipping

[0106] The invention is defined with reference to the following clauses:

Clause 1: An antisense molecule capable of binding to a selected target site to induce exon skipping in the dystrophin gene, as set forth in SEQ ID NO: 1 to 202.

Clause 2: An antisense molecule according to clause 1 capable of inducing exon skipping in exons 3, 4, 8, 10 to 16, 19 to 40, 42 to 44, 46, 47 and 50 to 53 of the dystrophin gene.

Clause 3: A combination of two or more antisense molecules according to clause 1 or clause 2 capable of binding to a selected target to induce exon skipping in the dystrophin gene.

Clause 4: A combination or two or more antisense molecules according to clause 3 selected from Table 1B.

Clause 5: A combination of two or more antisense molecules according to clause 1 or clause 2 joined together to form a "weasel", wherein said weasel is capable of binding to a selected target to induce exon skipping in the dystrophin gene.

Clause 6: A combination of two or more antisense molecules according to clause 5 selected from Table 1C.

Clause 7: The antisense molecule according to anyone of clauses 1 to 6, capable of binding to a selected target site, wherein the target site is an mRNA splicing site selected from a splicer donor site, splice acceptor sites or exonic splicing enhancer elements.

Clause 8: A method of treating muscular dystrophy in a patient comprising administering to the patient a composition comprising an antisense molecule according to anyone of clauses 1 to 6.

Clause 9: A pharmaceutical or therapeutic composition for the treatment of muscular dystrophy in a patient comprising (a) at least an antisense molecule according to anyone of clause 1 to 6, and (b) one or more pharmaceutically acceptable carriers and/or diluents.

Clause 10: The composition according to clause 9, comprising about 20 nM to 600 nM of the antisense molecule.

Clause 11. The use of an antisense molecule according to anyone of clauses 1 to 6 for the manufacture of a medicament for modulation of muscular dystrophy.

Clause 12. An antisense molecule according to anyone of clauses 1 to 6 for use in antisense molecule based therapy.

Clause 13. An antisense molecule according to anyone of clauses 1 to 6 as herein before described with reference to the examples.

Clause 14. A kit comprising at least one antisense molecule according to anyone of clauses 1 to 6, a suitable carrier and instructions for its use.

SEQUENCE LISTING

[0107]

<110> The University of Western Australia

<120> Antisense Oligonucleotides for Inducing Exon Skipping and Methods of Use Thereof

<130> AHB/FP6689632

<140> EP10004274.6
<141> 2005-06-28

<150> EP05754344.9
<151> 2005-06-28

<150> PCT/AU2005/000943
<151> 2005-06-28

<150> AU20040903474
<151> 2004-06-28

<160> 212

<170> PatentIn version 3.3

<210> 1
<211> 24
<212> RNA
<213> Human

<400> 1

gauagguggu aucaacaucu guaa 24

<210> 2
<211> 21
<212> RNA
<213> Human

<400> 2
gauagguggu aucaacaucu g 21

<210> 3
<211> 25
<212> RNA
<213> Human

<400> 3
gauagguggu aucaacaucu guaag 25

<210> 4
<211> 20
<212> RNA
<213> Human

<400> 4
ggugguauca acaucuguaa 20

<210> 5
<211> 20
<212> RNA
<213> Human

<400> 5
guaucaacau cuguaagcac 20

<210> 6
<211> 23
<212> RNA
<213> Human

<400> 6
ugcauguucc agucguugug ugg 23

<210> 7
<211> 25
<212> RNA
<213> Human

<400> 7
cacuauucca gucaaauagg ucugg 25

<210> 8
<211> 25
<212> RNA
<213> Human

<400> 8
auuuaccaac cuucaggauc gagua 25

<210> 9
<211> 21
<212> RNA
<213> Human

<400> 9
ggccuaaaac acauacacau a 21

<210> 10
<211> 20
<212> RNA
<213> Canine

<400> 10
cauuuuugac cuacaugugg 20

<210> 11
<211> 20
<212> RNA
<213> Canine

<400> 11
uuugaccuac auguggaaag 20

<210> 12
<211> 26
<212> RNA
<213> Canine

<400> 12
uacauuuuug accuacaugu ggaaag 26

<210> 13
<211> 22
<212> RNA
<213> Canine

<400> 13
auuuuugacc uacaugggaa ag 22

<210> 14
<211> 23
<212> RNA
<213> Canine

<400> 14
uacgaguuga uugucggacc cag 23

<210> 15
<211> 25
<212> RNA
<213> Canine

<400> 15
guggucuccu uaccuaugac ugugg 25

<210> 16
<211> 17

<212> RNA
<213> Canine

<400> 16
ggucuccuua ccuauga 17

<210> 17
<211> 24
<212> RNA
<213> Human

<400> 17
ugucucagua aucuucuuac cuau 24

<210> 18
<211> 24
<212> RNA
<213> Human

<400> 18
ucuuaccuau gacuauggau gaga 24

<210> 19
<211> 20
<212> RNA
<213> Human

<400> 19
gcaugaacuc uuguggaucc 20

<210> 20
<211> 20
<212> RNA
<213> Human

<400> 20
ccaggguacu acuuacauua 20

<210> 21
<211> 21
<212> RNA
<213> Human

<400> 21
aucgugugug acagcaucca g 21

<210> 22
<211> 30
<212> RNA
<213> Human

<400> 22
uguucagggc augaacucuu guggauccuu 30

<210> 23
<211> 31
<212> RNA
<213> Human

<400> 23
uaggaggcgc cucccauccu guaggucacu g 31

<210> 24
<211> 31
<212> RNA
<213> Human

<400> 24
aggucuagga ggcgccuccc auccuguagg u 31

<210> 25
<211> 25
<212> RNA
<213> Human

<400> 25
gcgccuccca uccuguaggu cacug 25

<210> 26
<211> 26
<212> RNA
<213> Human

<400> 26
cuucgaggag gucuaggagg cgccuc 26

<210> 27
<211> 21
<212> RNA
<213> Human

<400> 27
cucccauccu guaggucacu g 21

<210> 28
<211> 22
<212> RNA
<213> Human

<400> 28
uaccaguuuu ugcccuguca gg 22

<210> 29
<211> 26
<212> RNA
<213> Human

<400> 29
ucaauaugcu gcuucccaaa cugaaa 26

<210> 30
<211> 25
<212> RNA
<213> Human

<400> 30
cuaggaggcg ccucccaucc uguag 25

<210> 31
<211> 31
<212> RNA
<213> Human

<400> 31
uuaugauuuc caucuacgau gucaguacuu c 31

<210> 32
<211> 31
<212> RNA
<213> Human

<400> 32
cuuaccugcc aguggaggau uauauuccaa a 31

<210> 33
<211> 25
<212> RNA
<213> Human

<400> 33
caucaggauu cuuaccugcc agugg 25

<210> 34
<211> 25
<212> RNA
<213> Human

<400> 34
cgaugucagu acuuccaaua uucac 25

<210> 35
<211> 18
<212> RNA
<213> Human

<400> 35
accauucauc aggauucu 18

<210> 36
<211> 18
<212> RNA
<213> Human

<400> 36
accugccagu ggaggauu 18

<210> 37
<211> 27
<212> RNA
<213> Human

<400> 37
ccaauauuca cuaaaucaac cuguuaa 27

<210> 38
<211> 30

<212> RNA
<213> Human

<400> 38
caggauucuu accugccagu ggaggauuau 30

<210> 39
<211> 31
<212> RNA
<213> Human

<400> 39
acgaugucag uacuuccaau auucacuaaa u 31

<210> 40
<211> 31
<212> RNA
<213> Human

<400> 40
auuuccaucu acgaugucag uacuuccaau a 31

<210> 41
<211> 21
<212> RNA
<213> Human

<400> 41
caggagcuuc caaaugcugc a 21

<210> 42
<211> 29
<212> RNA
<213> Human

<400> 42
cuugucuuca ggagcuucca aaugcugca 29

<210> 43
<211> 22
<212> RNA
<213> Human

<400> 43
uccucagcag aaagaagcca cg 22

<210> 44
<211> 20
<212> RNA
<213> Human

<400> 44
uuagaaaucu cuccuugugc 20

<210> 45
<211> 20
<212> RNA
<213> Human

<400> 45
uaaauugggu guuacacaau 20

<210> 46
<211> 24
<212> RNA
<213> Human

<400> 46
cccugaggca uucccaucuu gaau 24

<210> 47
<211> 20
<212> RNA
<213> Human

<400> 47
aggacuuacu ugcuuuguuu 20

<210> 48
<211> 23
<212> RNA
<213> Human

<400> 48
cuugaauuua ggagauucau cug 23

<210> 49
<211> 23
<212> RNA
<213> Human

<400> 49
caucuucuga uaauuuuccu guu 23

<210> 50
<211> 24
<212> RNA
<213> Human

<400> 50
ucuucuguuu uuguuagcca guca 24

<210> 51
<211> 20
<212> RNA
<213> Human

<400> 51
ucuauguaaa cugaaaauuu 20

<210> 52
<211> 20
<212> RNA
<213> Human

<400> 52
uucuggagau ccauuaaaac 20

<210> 53
<211> 24
<212> RNA
<213> Human

<400> 53
cagcaguugc gugaucucca cuag 24

<210> 54
<211> 21
<212> RNA
<213> Human

<400> 54
uucaucaacu accaccacca u 21

<210> 55
<211> 25
<212> RNA
<213> Human

<400> 55
cuaagcaaaa uaaucugacc uuaag 25

<210> 56
<211> 28
<212> RNA
<213> Human

<400> 56
cuuguaaaag aacccagcgg ucuucugu 28

<210> 57
<211> 22
<212> RNA
<213> Human

<400> 57
caucuacaga uguuugccca uc 22

<210> 58
<211> 23
<212> RNA
<213> Human

<400> 58
gaaggauguc uuguaaaaga acc 23

<210> 59
<211> 20
<212> RNA
<213> Human

<400> 59
accuguucuu caguaagacg 20

<210> 60
<211> 24

<212> RNA
<213> Human

<400> 60
caugacacac cuguucuuca guaa 24

<210> 61
<211> 20
<212> RNA
<213> Human

<400> 61
cauuugagaa ggaugucuug 20

<210> 62
<211> 24
<212> RNA
<213> Human

<400> 62
aucucccaau accuggagaa gaga 24

<210> 63
<211> 31
<212> RNA
<213> Human

<400> 63
gccaugcacu aaaaaggcac ugcaagacau u 31

<210> 64
<211> 24
<212> RNA
<213> Human

<400> 64
ucuuuaaagc caguugugug aauc 24

<210> 65
<211> 21
<212> RNA
<213> Human

<400> 65
uuucugaaag ccaugcacua a 21

<210> 66
<211> 25
<212> RNA
<213> Human

<400> 66
guacauacgg ccaguuuuug aagac 25

<210> 67
<211> 31
<212> RNA
<213> Human

<400> 67
cuagauccgc uuuuaaaacc uguuaaaaca a 31

<210> 68
<211> 31
<212> RNA
<213> Human

<400> 68
ucuuuucuag auccgcuuuu aaaaccuguu a 31

<210> 69
<211> 25
<212> RNA
<213> Human

<400> 69
cuagauccgc uuuuaaaacc uguua 25

<210> 70
<211> 23
<212> RNA
<213> Human

<400> 70
ccgucuucug ggucacugac uua 23

<210> 71
<211> 26
<212> RNA
<213> Human

<400> 71
cuagauccgc uuuuaaaacc uguuaa 26

<210> 72
<211> 20
<212> MRNA
<213> Human

<400> 72
ccgcuuuuaa aaccuguuaa 20

<210> 73
<211> 26
<212> RNA
<213> Human

<400> 73
uggauugcuu uuucuuuucu agaucc 26

<210> 74
<211> 25
<212> RNA
<213> Human

<400> 74
caugcuuccg ucuucugggu cacug 25

<210> 75
<211> 23
<212> RNA
<213> Human

<400> 75
gaucuuguuu gagugaauac agu 23

<210> 76
<211> 22
<212> RNA
<213> Human

<400> 76
guuauccagc caugcuuccg uc 22

<210> 77
<211> 25
<212> RNA
<213> Human

<400> 77
ugauaauugg uaucacuaac cugug 25

<210> 78
<211> 22
<212> RNA
<213> Human

<400> 78
guaucacuaa ccugugcugu ac 22

<210> 79
<211> 20
<212> RNA
<213> Human

<400> 79
cagcaguagu ugucaucugc 20

<210> 80
<211> 31
<212> RNA
<213> Human

<400> 80
gccugagcug aucugcuggc aucuugcagu u 31

<210> 81
<211> 28
<212> RNA
<213> Human

<400> 81
cuggcagaau ucgauccacc ggcuguuc 28

<210> 82
<211> 22

<212> RNA
<213> Human

<400> 82
cagcaguagu ugucaucugc uc 22

<210> 83
<211> 19
<212> RNA
<213> Human

<400> 83
ugauggggug guggguugg 19

<210> 84
<211> 25
<212> RNA
<213> Human

<400> 84
aucugcauua acacccucua gaaag 25

<210> 85
<211> 24
<212> RNA
<213> Human

<400> 85
ccggcuguuc aguuguucug aggc 24

<210> 86
<211> 28
<212> RNA
<213> Human

<400> 86
aucugcauua acacccucua gaaagaaa 28

<210> 87
<211> 28
<212> RNA
<213> Human

<400> 87
gaaggagaag agauucuuac cuuacaaa 28

<210> 88
<211> 20
<212> RNA
<213> Human

<400> 88
auucgaucca ccggcuguuc 20

<210> 89
<211> 19
<212> RNA
<213> Human

<400> 89
cugcuggcau cuugcaguu 19

<210> 90
<211> 22
<212> DNA
<213> Human

<400> 90
gccgguugac uucauccugu gc 22

<210> 91
<211> 22
<212> RNA
<213> Human

<400> 91
cugcauccag gaacaugggu cc 22

<210> 92
<211> 23
<212> RNA
<213> Human

<400> 92
gucugcaucc aggaacaugg guc 23

<210> 93
<211> 24
<212> DNA
<213> Human

<400> 93
guugaagauc ugauagccgg uuga 24

<210> 94
<211> 24
<212> RNA
<213> Human

<400> 94
uacuuacugu cuguagcucu uucu 24

<210> 95
<211> 24
<212> RNA
<213> Human

<400> 95
cacucauggu cuccugauag cgca 24

<210> 96
<211> 22
<212> RNA
<213> Human

<400> 96
cugcaauucc ccgagucucu gc 22

<210> 97
<211> 23
<212> RNA
<213> Human

<400> 97
acugcuggac ccauguccug aug 23

<210> 98
<211> 21
<212> RNA
<213> Human

<400> 98
cuaaguugag guauggagag u 21

<210> 99
<211> 23
<212> RNA
<213> Human

<400> 99
uauucacaga ccugcaauuc ccc 23

<210> 100
<211> 26
<212> RNA
<213> Human

<400> 100
acaguggugc ugagauagua uaggcc 26

<210> 101
<211> 22
<212> RNA
<213> Human

<400> 101
uaggccacuu uguugcucuu gc 22

<210> 102
<211> 19
<212> RNA
<213> Human

<400> 102
uucagagggc gcuuucuuc 19

<210> 103
<211> 23
<212> DNA
<213> Human

<400> 103
gggcaggcca uucccuccuuc aga 23

<210> 104
<211> 24

<212> RNA
<213> Human

<400> 104
ucuucagggu uuguauguga uucu 24

<210> 105
<211> 27
<212> RNA
<213> Human

<400> 105
cugggcugaa uugucugaau aucacug 27

<210> 106
<211> 26
<212> RNA
<213> Human

<400> 106
cuguuggcac augugauccc acugag 26

<210> 107
<211> 24
<212> RNA
<213> Human

<400> 107
gucuauaccu guuggcacau guga 24

<210> 108
<211> 25
<212> RNA
<213> Human

<400> 108
ugcuuucugu aauucaucug gaguu 25

<210> 109
<211> 26
<212> RNA
<213> Human

<400> 109
ccuccuuucu ggcauagacc uuccac 26

<210> 110
<211> 25
<212> RNA
<213> Human

<400> 110
ugugucaucc auucgugcau cucug 25

<210> 111
<211> 25
<212> RNA
<213> Human

<400> 111
uuaaggccuc uugugcuaca ggugg 25

<210> 112
<211> 23
<212> MRNA
<213> Human

<400> 112
gggccucuuc uuuagcucuc uga 23

<210> 113
<211> 22
<212> RNA
<213> Human

<400> 113
gacuuccaaa gucuugcauu uc 22

<210> 114
<211> 24
<212> RNA
<213> Human

<400> 114
gccaacaugc ccaaacuucc uaag 24

<210> 115
<211> 26
<212> RNA
<213> Human

<400> 115
cagagauuuc cucagcuccg ccagga 26

<210> 116
<211> 21
<212> RNA
<213> Human

<400> 116
cuuacaucua gcaccucaga g 21

<210> 117
<211> 25
<212> RNA
<213> Human

<400> 117
uccgccaucu guuagggucu gugcc 25

<210> 118
<211> 25
<212> RNA
<213> Human

<400> 118
auuuggguua uccucugaau gucgc 25

<210> 119
<211> 22
<212> RNA
<213> Human

<400> 119
cauaccucuu cauguaguuc uc 22

<210> 120
<211> 31
<212> RNA
<213> Human

<400> 120
cauuugagcu gcguccaccu ugucgucugu g 31

<210> 121
<211> 26
<212> RNA
<213> Human

<400> 121
uccugggcag acuggaugcu cuguuc 26

<210> 122
<211> 23
<212> RNA
<213> Human

<400> 122
uugccugggc uuccugaggc auu 23

<210> 123
<211> 24
<212> RNA
<213> Human

<400> 123
uucugaaaua acauauaccu gugc 24

<210> 124
<211> 25
<212> RNA
<213> Human

<400> 124
uaguuucuga aauaacauau accug 25

<210> 125
<211> 21
<212> RNA
<213> Human

<400> 125
gacuugucaa aucagauugg a 21

<210> 126
<211> 24

<212> RNA
<213> Human

<400> 126
guuucugaaa uaacauauac cugu 24

<210> 127
<211> 20
<212> RNA
<213> Human

<400> 127
caccagaaau acauaccaca 20

<210> 128
<211> 20
<212> RNA
<213> Human

<400> 128
caaugauuua gcugugacug 20

<210> 129
<211> 23
<212> RNA
<213> Human

<400> 129
cgaaacuuca uggagacauc uug 23

<210> 130
<211> 25
<212> RNA
<213> Human

<400> 130
cuuguagacg cugcucaaaa uuggc 25

<210> 131
<211> 20
<212> RNA
<213> Human

<400> 131
caugcacaca ccuuugcucc 20

<210> 132
<211> 24
<212> RNA
<213> Human

<400> 132
ucuguacaau cugacgucca gucu 24

<210> 133
<211> 27
<212> RNA
<213> Human

<400> 133
gucuuuauca ccauuuccac uucagac 27

<210> 134
<211> 25
<212> RNA
<213> Human

<400> 134
ccgucugcuu uuucuguaca aucug 25

<210> 135
<211> 22
<212> RNA
<213> Human

<400> 135
uccauaucug uagcugccag cc 22

<210> 136
<211> 23
<212> RNA
<213> Human

<400> 136
ccaggcaacu ucagaaucca aau 23

<210> 137
<211> 30
<212> RNA
<213> Human

<400> 137
uuucuguuac cugaaaagaa uuauaaugaa 30

<210> 138
<211> 25
<212> RNA
<213> Human

<400> 138
cauucauuuc cuuucgcauc uuacg 25

<210> 139
<211> 26
<212> RNA
<213> Human

<400> 139
ugaucucuuu gucaauucca uaucug 26

<210> 140
<211> 27
<212> RNA
<213> Human

<400> 140
uucagugaua uagguuuuac cuuuccc 27

<210> 141
<211> 26
<212> DNA
<213> Human

<400> 141
cuguagcugc cagccauucu gucaag 26

<210> 142
<211> 21
<212> RNA
<213> Human

<400> 142
ucuucugcuc gggaggugac a 21

<210> 143
<211> 20
<212> RNA
<213> Human

<400> 143
ccaguuacua uucagaagac 20

<210> 144
<211> 20
<212> RNA
<213> Human

<400> 144
ucuucaggug caccuucugu 20

<210> 145
<211> 25
<212> RNA
<213> Human

<400> 145
ugugaugugg uccacauucu gguca 25

<210> 146
<211> 20
<212> RNA
<213> Human

<400> 146
ccauguguuu cugguauucc 20

<210> 147
<211> 25
<212> RNA
<213> Human

<400> 147
cguguagagu ccaccuuugg gcgua 25

<210> 148
<211> 24

<212> RNA
<213> Human

<400> 148
uacuaauuuc cugcaguggu cacc 24

<210> 149
<211> 24
<212> RNA
<213> Human

<400> 149
uucuguguga aauggcugca aauc 24

<210> 150
<211> 20
<212> RNA
<213> Human

<400> 150
ccuucaaagg aauggaggcc 20

<210> 151
<211> 25
<212> RNA
<213> Human

<400> 151
ugcugaauuu cagccuccag ugguu 25

<210> 152
<211> 25
<212> RNA
<213> Human

<400> 152
ugaagucuuc cucuuucaga uucac 25

<210> 153
<211> 24
<212> RNA
<213> Human

<400> 153
cuggcuuucu cucaucugug auuc 24

<210> 154
<211> 20
<212> RNA
<213> Human

<400> 154
guuguaaguu gucuccucuu 20

<210> 155
<211> 20
<212> RNA
<213> Human

<400> 155
uugucuguaa cagcugcugu 20

<210> 156
<211> 20
<212> RNA
<213> Human

<400> 156
gcucuaauac cuugagagca 20

<210> 157
<211> 22
<212> RNA
<213> Human

<400> 157
cuuugagacc ucaaauccug uu 22

<210> 158
<211> 25
<212> RNA
<213> Human

<400> 158
cuuuauuuuc cuuucaucuc ugggc 25

<210> 159
<211> 27
<212> RNA
<213> Human

<400> 159
aucguuucuu cacggacagu gugcugg 27

<210> 160
<211> 24
<212> RNA
<213> Human

<400> 160
gggcuuguga gacaugagug auuu 24

<210> 161
<211> 22
<212> RNA
<213> Human

<400> 161
accuucagag gacuccucuu gc 22

<210> 162
<211> 25
<212> RNA
<213> Human

<400> 162
uauguguuac cuacccuugu cgguc 25

<210> 163
<211> 20
<212> RNA
<213> Human

<400> 163
ggagagagcu uccuguagcu 20

<210> 164
<211> 23
<212> RNA
<213> Human

<400> 164
ucacccuuuc cacaggcguu gca 23

<210> 165
<211> 20
<212> RNA
<213> Human

<400> 165
uuugugucuu ucugagaaac 20

<210> 166
<211> 20
<212> RNA
<213> Human

<400> 166
aaagacuuac cuuaagauac 20

<210> 167
<211> 20
<212> RNA
<213> Human

<400> 167
aucugucaaa ucgccugcag 20

<210> 168
<211> 20
<212> RNA
<213> Human

<400> 168
uuaccuugac uugcucaagc 20

<210> 169
<211> 20
<212> MRNA
<213> Human

<400> 169
uccagguuca agugggauac 20

<210> 170
<211> 25

<212> RNA
<213> Human

<400> 170
gcucuucugg gcuuauggga gcacu 25

<210> 171
<211> 27
<212> RNA
<213> Human

<400> 171
accuuuaucc acuggagauu ugucugc 27

<210> 172
<211> 21
<212> RNA
<213> Human

<400> 172
uuccaccagu aacugaaaca g 21

<210> 173
<211> 29
<212> RNA
<213> Human

<400> 173
ccacucagag cucagaucuu cuaacuucc 29

<210> 174
<211> 27
<212> RNA
<213> Human

<400> 174
cuucaacuca gagcucagau cuucuaa 27

<210> 175
<211> 25
<212> RNA
<213> Human

<400> 175
gggauccagu auacuuacag gcucc 25

<210> 176
<211> 26
<212> RNA
<213> Human

<400> 176
accagaguaa cagucugagu aggagc 26

<210> 177
<211> 23
<212> DNA
<213> Human

<400> 177
cucauaccuu cugcuugaug auc 23

<210> 178
<211> 24
<212> RNA
<213> Human

<400> 178
uucuguccaa gcccgguuga aauc 24

<210> 179
<211> 25
<212> RNA
<213> Human

<400> 179
acaucaagga ggauggcauu ucuag 25

<210> 180
<211> 25
<212> RNA
<213> Human

<400> 196
acaucaagga agauggcauu ucuag 25

<210> 181
<211> 30
<212> RNA
<213> Human

<400> 181
cuccaacauc aaggaagaug gcauuucuag 30

<210> 182
<211> 25
<212> RNA
<213> Human

<400> 182
aucauuuuuu cucauaccuu cugcu 25

<210> 183
<211> 36
<212> DNA
<213> Human

<400> 183
aucauuuuuu cucauaccuu cugcuaggag cuaaaa 36

<210> 184
<211> 21
<212> RNA
<213> Human

<400> 184
cacccaccau cacccucugu g 21

<210> 185
<211> 22
<212> RNA
<213> Human

<400> 185
aucaucucgu ugauauccue aa 22

<210> 186
<211> 21
<212> RNA
<213> Human

<400> 186
uccugcauug uugccuguaa g 21

<210> 187
<211> 30
<212> RNA
<213> Human

<400> 187
uccaacuggg gacgccucug uuccaaaucc 30

<210> 188
<211> 21
<212> RNA
<213> Human

<400> 188
acuggggacg ccucuguucc a 21

<210> 189
<211> 20
<212> RNA
<213> Human

<400> 189
ccguaaugau uguucuagcc 20

<210> 190
<211> 20
<212> RNA
<213> Human

<400> 190
uguuaaaaaa cuuacuucga 20

<210> 191
<211> 25
<212> RNA
<213> Human

<400> 191
cauucaacug uugccuccgg uucug 25

<210> 192
<211> 24

<212> RNA
<213> Human

<400> 192
cuguugccuc cgguucugaa ggug 24

<210> 193
<211> 31
<212> RNA
<213> Human

<400> 193
cauucaacug uugccuccgg uucugaaggu g 31

<210> 194
<211> 21
<212> RNA
<213> Human

<400> 194
uacuaaccuu gguuucugug a 21

<210> 195
<211> 25
<212> RNA
<213> Human

<400> 195
cugaaggugu ucuuguacuu caucc 25

<210> 196
<211> 27
<212> RNA
<213> Human

<400> 196
uguauaggga cccuccuucc augacuc 27

<210> 197
<211> 25
<212> RNA
<213> Human

<400> 197
cuaaccuugg uuucugugau uuucu 25

<210> 198
<211> 27
<212> RNA
<213> Human

<400> 198
gguaucuuug auacuaaccu ugguuuc 27

<210> 199
<211> 22
<212> RNA
<213> Human

<400> 199
auucuuucaa cuagaauaaa ag 22

<210> 200
<211> 25
<212> RNA
<213> Human

<400> 200
gauucugaau ucuuucaacu agaau 25

<210> 201
<211> 20
<212> RNA
<213> Human

<400> 201
aucccacuga uucugaauuc 20

<210> 202
<211> 22
<212> RNA
<213> Human

<400> 202
uuggcucugg ccuguccuaa ga 22

<210> 203
<211> 29
<212> RNA
<213> Human

<400> 203
cucuuuuccag guucaagugg gauacuagc 30

<210> 204
<211> 31
<212> RNA
<213> Human

<400> 204
caagcuuuuc uuuuaguugc ugcucuuuuc c 31

<210> 205
<211> 30
<212> RNA
<213> Human

<400> 205
uauucuuuug uucuucuagc cuggagaaag 30

<210> 206
<211> 28
<212> RNA
<213> Human

<400> 206
cugcuuccuc caaccauaaa acaaauuc 28

<210> 207
<211> 26
<212> RNA
<213> Human

<400> 207
ccaaugccau ccuggaguuc cuguaa 26

<210> 208
<211> 20
<212> RNA
<213> Human

<400> 208
uccuguagaa uacuggcauc 20

<210> 209
<211> 27
<212> RNA
<213> Human

<400> 209
ugcagaccuc cugccaccgc agauuca 27

<210> 210
<211> 20
<212> RNA
<213> Human

<400> 210
cuaccucuuu uuucugucug 20

<210> 211
<211> 20
<212> RNA
<213> Human

<400> 211
uguuuuugag gauugcugaa 20

<210> 212
<211> 84
<212> MRNA
<213> Human

<400> 212

cagcaguagu ugucaucugc ucaacuggca gaauucgauc caccggcugu ucaagccuga 60
gcugaucugc ucgcaucuug cagu 84

Claims

1. An isolated antisense oligonucleotide that binds to human dystrophin pre-mRNA wherein said oligonucleotide is 20 to 31 nucleotides in length and is an oligonucleotide that is specifically hybridizable to an exon 53 target region of the Dystrophin gene designated as annealing site H53A (+23+47), annealing site H53A (+39+69), or both,

wherein said antisense oligonucleotide is a morpholino antisense oligonucleotide, and,
wherein said oligonucleotide induces exon 53 skipping.

2. An antisense oligonucleotide according to claim 1 selected from SEQ ID NOS: 192, 193 and 195, optionally wherein the uracil bases (U) are thymine bases (T).

3. The antisense oligonucleotide of claim 1 or 2, wherein the oligonucleotide is chemically linked to one or more moieties or conjugates that enhance the activity, cellular distribution, or cellular uptake of the antisense oligonucleotide.

4. The antisense oligonucleotide of claim 3, wherein the oligonucleotide is chemically linked to a polyethylene glycol chain.

5. The antisense oligonucleotide of claim 1 or 2 comprising a 5-substituted pyrimidine base.

6. The antisense oligonucleotide of claim 1 or 2 comprising a 5-methylcytosine base.

7. A composition, comprising an antisense oligonucleotide of any one of claims 1-6 and a saline solution that includes a phosphate buffer.

8. An antisense oligonucleotide of any one of claims 1-6, or a composition of claim 7, for use in a method of treatment of muscular dystrophy.

9. The antisense oligonucleotide or composition for use according to claim 8, wherein the muscular dystrophy is Duchenne Muscular Dystrophy.

Patentansprüche

1. Isoliertes Antisense-Oligonucleotid, das an menschliche Dystrophin-Prä-mRNA bindet, worin das Oligonucleotid 20 bis 31 Nucleotide lang ist und ein Oligonucleotid ist, das spezifisch an eine Exon-53-Target-Region des Dystrophin-Gens hybridisierbar ist, die als Annealing-Stelle H53A (+23+47), Annealing-Stelle H53A (+39+69) oder beides bezeichnet wird,
worin das Antisense-Oligonucleotid ein Morpholino-Antisense-Oligonucleotid ist und
worin das Oligonucleotid ein Überspringen von Exon 53 induziert.

2. Antisense-Oligonucleotid nach Anspruch 1, ausgewählt aus den SEQ-ID Nr. 192, 193 und 195, worin die Uracil-Basen (U) gegebenenfalls Thymin-Basen (T) sind.

3. Antisense-Oligonucleotid nach Anspruch 1 oder 2, worin das Oligonucleotid chemisch an eine oder mehrere Gruppierungen oder Konjugate gebunden ist, die die Aktivität, Zellverteilung oder Zellaufnahme des Antisense-Oligonucleotids verbessern.

4. Antisense-Oligonucleotid nach Anspruch 3, worin das Oligonucleotid chemisch an eine Polyethylenglykol-Kette gebunden ist.

5. Antisense-Oligonucleotid nach Anspruch 1 oder 2, umfassend eine 5-substituierte Pyrimidin-Base.

6. Antisense-Oligonucleotid nach Anspruch 1 oder 2, umfassend eine 5-Methylcytosin-Base.

7. Zusammensetzung, umfassend ein Antisense-Oligonucleotid nach einem der Ansprüche 1-6 und eine Kochsalzlösung, die einen Phosphatpuffer umfasst.

8. Antisense-Oligonucleotid nach einem der Ansprüche 1-6 oder Zusammensetzung nach Anspruch 7 zur Verwendung in einem Verfahren zur Behandlung von Muskeldystrophie.

9. Antisense-Oligonucleotid oder Zusammensetzung zur Verwendung nach Anspruch 8, worin die Muskeldystrophie eine Muskeldystrophie Duchenne ist.

Revendications

1. Oligonucléotide antisens isolé qui se lie à un pré-ARNm de la dystrophine humaine, où ledit oligonucléotide possède une longueur de 20 à 31 nucléotides et est un oligonucléotide qui peut spécifiquement s'hybrider à une région cible de l'exon 53 du gène de la dystrophine désignée en tant que site d'annelage H53A (+23+47), site d'annelage H53A (+39+69), ou les deux,
où ledit oligonucléotide antisens est un oligonucléotide antisens morpholino, et,
où ledit oligonucléotide induit un saut de l'exon 53.

2. Oligonucléotide antisens selon la revendication 1 sélectionné parmi SEQ ID NO: 192, 193 et 195, facultativement où les bases uracile (U) sont des bases thymine (T).

3. Oligonucléotide antisens selon la revendication 1 ou 2, où l'oligonucléotide est chimiquement lié à un ou plusieurs fragments ou conjugués qui améliorent l'activité, la distribution cellulaire, ou l'absorption cellulaire de l'oligonucléotide antisens.

4. Oligonucléotide antisens selon la revendication 3, où l'oligonucléotide est chimiquement lié à une chaîne polyéthylène glycol.

5. Oligonucléotide antisens selon la revendication 1 ou 2, comprenant une base pyrimidine 5-substituée.

6. Oligonucléotide antisens selon la revendication 1 ou 2, comprenant une base 5-méthylcytosine.

7. Composition, comprenant un oligonucléotide antisens selon l'une quelconque des revendications 1-6 et une solution saline qui comprend un tampon phosphate.

8. Oligonucléotide antisens selon l'une quelconque des revendications 1-6, ou composition selon la revendication 7, destiné(e) à être utilisé(e) dans un procédé de traitement de la dystrophie musculaire.

9. Oligonucléotide antisens ou composition destiné(e) à être utilisé(e) selon la revendication 8, où la dystrophie musculaire est la dystrophie musculaire de Duchenne.

FIGURE 1.

bp Acceptor ESE Donor

ucaugcacugagugaccucuuucucgcagGCGCUAGCUGGAGCA/////CCGUGCAGACUGACGgucucau

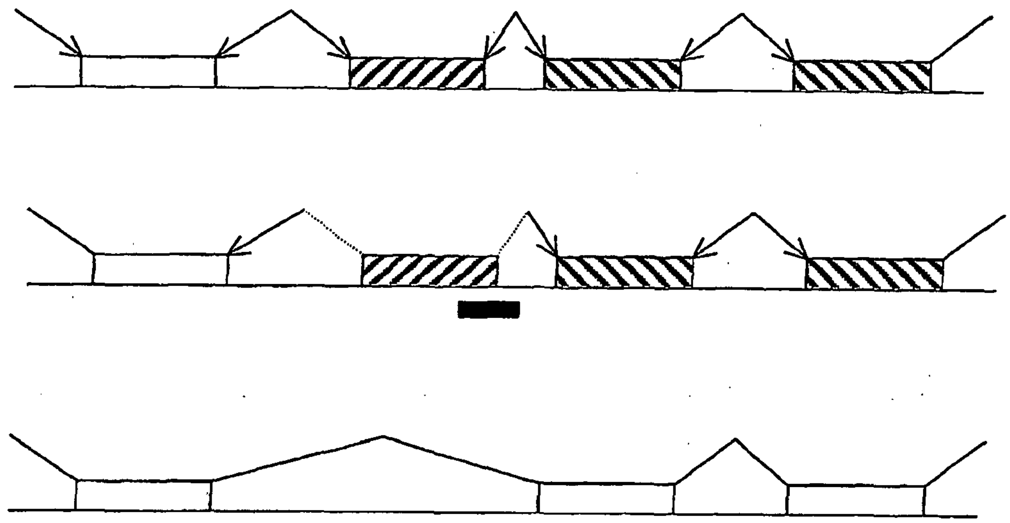
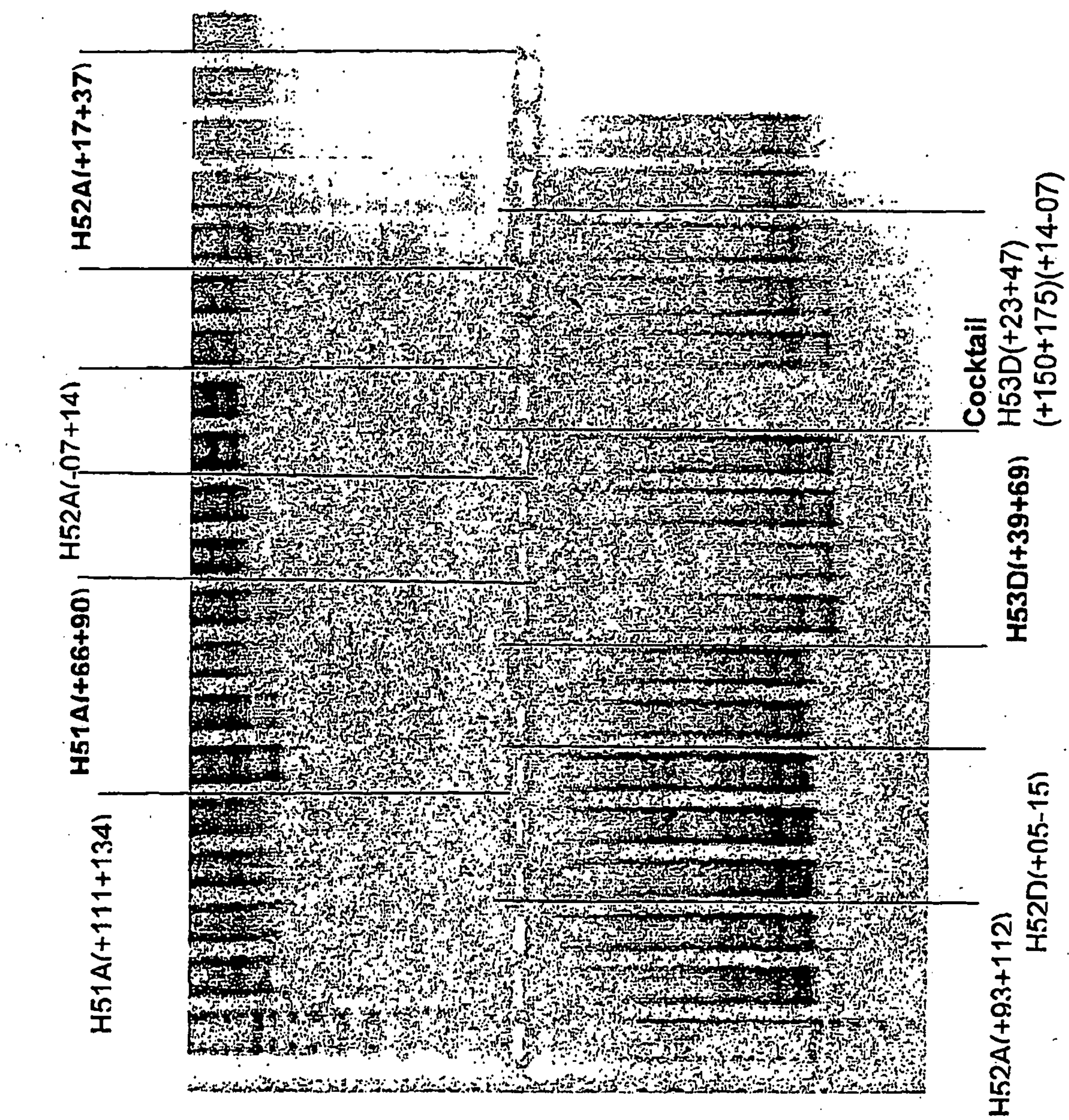


FIGURE 2

FIGURE 3


H51A(+111+134)
H51A(+66+90)
H52A(-07+14)
H52A(+17+37)
H52A(+93+112)
H52D(+05-15)
H53D(+39+69)
Cocktail
H53D(+23+47)
(+150+175)(+14-07)

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- CA 2507125 **[0016]**
- WO 2004083446 A **[0016]**
- US 5149797 A **[0059]**
- US 4458066 A **[0066]**
- US 20040248833 A **[0075]**
- US 6806084 B **[0077]**
- EP 10004274 A **[0107]**
- EP 05754344 A **[0107]**
- AU 2005000943 W **[0107]**
- AU 20040903474 **[0107]**

Non-patent literature cited in the description

- **SIERAKOWSKA H et al.** *Proc Natl Acad Sci USA,* 1996, vol. 93, 12840-12844 **[0004]**
- **WILTON SD et al.** *Neuromusc Disorders,* 1999, vol. 9, 330-338 **[0004]**
- **VAN DEUTEKOM JC et al.** *Human Mol Genet,* 2001, vol. 10, 1547-1554 **[0004]**
- **SHERRAT TG et al.** *Am J Hum Genet,* 1993, vol. 53, 1007-1015 **[0006]**
- **LU QL et al.** *Nature Medicine,* 2003, vol. 9, 1009-1014 **[0006]**
- **AARTSMA-RUS A et al.** *Am J Hum Genet,* 2004, vol. 74, 83-92 **[0006]**
- **MATSUO et al.** *J Clin Invest.,* 1991, vol. 87, 2127-2131 **[0009]**
- **TAKESHIMA et al.** *J. Clin. Invest.,* 1995, vol. 95, 515-520 **[0009]**
- **DUNCKLEY et al.** *Nucleosides & Nucleotides,* 1997, vol. 16, 1665-1668 **[0010]**
- **DUNCKLEY et al.** *Human Mol. Genetics,* 1998, vol. 5, 1083-90 **[0011]**
- **ERRINGTON et al.** *J Gen Med,* 2003, vol. 5, 518-527 **[0012]**
- **WILTON et al.** *Neuromuscular Disorders,* 1999, vol. 9, 330-338 **[0014]**
- **MANN CJ et al.** *J Gen Med,* 2002, vol. 4, 644-654 **[0015]**
- **AARTSMA-RUS et al.** *Human Molecular Genetics,* 2003, vol. 12, 907-14 **[0016]**
- **AARTSMA-RUS et al.** *Neuromuscular Disorders,* 2002, vol. 12, 71-7 **[0016]**
- **MANN et al.** *J Gen Med,* 2002, vol. 4, 644-654 **[0034]**
- Improved antisense oligonucleotide induced exon skipping in the mdx mouse model of muscular dystrophy. *J Gen Med,* vol. 4, 644-654 **[0046]**
- **BEAUCAGE et al.** *Tetrahedron Letters,* 1981, vol. 22, 1859-1862 **[0067]**
- **MARTIN.** Remington's Pharmaceutical Sciences. Mack Publishing Co, 1990 **[0071]**
- **MARTIN.** Remington's Pharmaceutical Sciences. Mack Publishing Co, 1990, 1435-1712 **[0072]**
- **MANN CJ et al.** Antisense-induced exon skipping and the synthesis of dystrophin in the mdx mouse. *Proc., Natl. Acad. Science,* 2001, vol. 98 (1), 42-47 **[0076]**
- **GEBSKI et al.** *Human Molecular Genetics,* 2003, vol. 12 (15), 1801-1811 **[0076]**
- **FRALEY et al.** *Trends Biochem. Sci.,* 1981, vol. 6, 77 **[0079]**
- **MANNINO et al.** *Biotechniques,* 1988, vol. 6, 682 **[0080]**
- **FRIEDMANN.** *Science,* 1989, vol. 244, 1275-1280 **[0083]**
- **ROSENBERG.** *Cancer Research,* 1991, vol. 51 (18), 5074S-5079S **[0084]**
- **ROSENFELD et al.** *Cell,* 1991, vol. 68, 143-155 **[0084]**
- **ROSENFELD et al.** *Science,* 1991, vol. 252, 431-434 **[0084]**
- **BRIGHAM et al.** *Am. J. Med. Sci.,* 1989, vol. 298, 278-281 **[0084]**
- **NABEL et al.** *Science,* 1990, vol. 249, 1285-1288 **[0084]**
- **HAZINSKI et al.** *Am. J. Resp. Cell Molec. Biol.,* 1991, vol. 4, 206-209 **[0084]**
- **WANG ; HUANG.** *Proc. Natl. Acad. Sci. (USA),* 1987, vol. 84, 7851-7855 **[0084]**
- **WU ; WU.** *J. Biol. Chem.,* 1988, vol. 263, 14621-14624 **[0084]**
- **WOLFF et al.** *Science,* 1990, vol. 247, 1465-1468 **[0084]**
- *Am. J. Med. Sci.,* 1989, vol. 298, 278-281 **[0084]**
- *Clinical Research,* 1991, 39 **[0084]**
- **ANDERSON.** *Science,* 1992, vol. 256, 808-813 **[0084]**
- **SAMBROOK et al.** Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, 1989 **[0093]**
- DNA Cloning: A Practical Approach. MRL Press, Ltd, 1985 **[0093]**

- **AUSUBEL, F. ; BRENT, R. ; KINGSTON, R.E. ; MOORE, D.D. ; SEIDMAN, J.G. ; SMITH, J.A. ; STRUHL, K.** Current Protocols in Molecular Biology. Greene Publishing Associates/Wiley Intersciences, 2002 **[0093]**

EXHIBIT AU

LEDERER & KELLER

Patentanwälte Partnerschaft mbB
European Patent and Trademark Attorneys

EPO - Munich
82

25. Aug. 2016

DR. A. VAN DER WERTH
(1934 – 1974)

DR. FRANZ LEDERER
(1963 – 2012)

DR. GÜNTER KELLER
Dipl.-Biol.

DR. MICHAEL BEST
Dipl.-Chem.

DR. STEPHAN TEIPEL
Dipl.-Chem.

DR. GEORG KALHAMMER
Dipl.-Biochem.

80538 MÜNCHEN
Unsöldstraße 2
Telefon +49 89 21 23 99 0
Telefax +49 89 21 23 99 22
E-Mail info@lederer-keller.de

European Patent Office

80298 Munich

Zur Kasse

Your reference	Our reference	25 August 2016
	ESP00349NIP	K/sh

Re.: European Patent No. 2 206 781 B1
European Patent Application No. 10004274.6
Patentee: The University of Western Australia
Opponent: Nippon Shinyaku Co., Ltd.

On behalf of

Nippon Shinyaku Co. Ltd.
14, Nishinosho-Monguchi-cho, Kisshoin,
Minami-ku, Kyoto-shi,
Kyoto 601-8550
Japan

OPPOSITION

is lodged according to Article 99 EPC against the above-referenced patent titled

"Antisense oligonucleotides for inducing exon skipping and methods for use thereof".

The opposition fee amounting to EUR 785,-- is to be debited from our deposit account no. 28000381. A corresponding payment order is enclosed.

The opponent has appointed us as his representatives and it is requested to effect all notifications to our address.

The European patent EP 2 206 781 is opposed in its full extent (claims 1 to 9).

The opposition is based on the grounds of Article 100(a), 100(b) and 100(c) EPC. In particular it is submitted that the patent lacks inventive step. It does not disclose the invention in a manner sufficiently clear and complete for it to be carried out by the skilled person and its subject matter extends beyond the content of the application as originally filed.

It is requested to revoke the European patent in total. Oral proceedings in accordance with Article 116 EPC are requested in the event that the Opposition Division does not reach the decision to revoke the patent on the basis of the written submission of the opponent.

Detailed statement of the grounds for opposition

I. The cited prior art

For substantiation of the opposition it is referred to the prior art documents cited in the opposed patent and during the granting proceedings and the following documents. In particular it is referred to the following documents:

D1 Corey et al., Genome Biology, 2001, 2(5) 1015.1-1015.3

D2 AU 2004903474 (priority document)

D3 WO 2004/083432

D4 WO 2004/048570 (≙ EP 1 568 769)

D5 CA 2 507 125

D6 Aartsma-Rus et al., Human Molecular Genetics (2003), vol. 12, no. 8; pp 907-914

D7 Aartsma-Rus et al., Neuromuscular Disorders vol.12,S71-S77(2002)

D8 experimental report

II. The subject matter of EP 2 206 781

Claim 1 of the opposed patent relates to

a) an isolated antisense oligonucleotide that
   - a_1) binds to human dystrophin pre-mRNA,
   - a_2) wherein said oligonucleotide is 20 to 31 nucleotides in length and

b) is an oligonucleotide that is specifically hybridizable to
   - b_1) an exon 53 target region of the Dystrophin gene designated as annealing site H53A (+23+47),
   - b_2) annealing site H53A (+39+69), or
   - b_3) both

c) wherein said antisense oligonucleotide is a morpholino antisense oligonucleotide and

d) wherein said oligonucleotide induces exon 53 skipping.

Claim 2 relates to an oligonucleotide according to claim 1 which is selected from SEQ ID NOs: 192, 193 and 195, optionally wherein the uracil bases (U) are thymine bases (T).

Claim 3 is referred back to claims 1 or 2 and defines the oligonucleotide further in that it is chemically linked to one or more moieties or conjugates that enhance the activity, cellular distribution, or cellular uptake of the antisense oligonucleotide.

Claim 4 is referred back to claim 3 and defines the moieties or conjugates wherein the oligonucleotide is chemically linked to a polyethylene glycol chain.

Claim 5 is referred back to claim 1 or 2 and mentions that it comprises a 5-substituted pyrimidine base.

Claim 6 is referred back to claim 1 or 2 and mentions that it comprises a 5-methylcytosine base.

Claim 7 relates to a composition comprising an oligonucleotide as defined in any of claims 1-6 and a saline solution that includes a phosphate buffer.

Claim 8 relates to an antisense oligonucleotide according to any of claims 1-6, or a composition of claim 7, for use in a method of treatment of muscular dystrophy.

Claim 9 is referred back to claim 8 and defines the muscular dystrophy further in that it is Duchenne Muscular Dystrophy.

It is alleged that the opposed patent provides a target region within exon 53 spanning the annealing sites H53A (+23+47) and/or H53A (+39+69) with a morpholino antisense oligonucleotide ("PMO") of 20 to 31 nucleotides in length. This target region is efficient in vivo splicing (compare with patentee's submission dated September 17, 2014). It is alleged that the key region of exon 53 can be targeted to provide therapeutically effective exon skipping.

III. Article 100(c)/123(2) EPC / added matter

3.1 <u>Intermediate generalization</u>

The granted claims have to be revoked in view of an inadmissible intermediate generalization since by the amendments features were taken out of the initial context and combined with others. Such "intermediate generalization" is considered to be inadmissible (see e.g. T962/98, T1408/04, T461/05 or T1118/10 to name only a few of the relevant decisions).

3.1.1 Three annealing sites

Claim 1 contains three annealing sites, namely

1) H53A (+23+47) which corresponds to feature b_1) and
2) annealing site H53A (+39+69) as annealing site b_2) and furthermore
3) both annealing sites (feature b_3)).

First of all it should be mentioned that feature b_3), namely both annealing sites, is obviously nowhere disclosed. To be more precise, the word "both" in combination with the two annealing sites representing features b_1) and b_2) of claim 1 can nowhere be found in the specification as originally filed (WO 2006/000057).

For the interpretation what the term "both" should mean according to the interpretation of patentee we refer to the submission of patentee's representatives dated September 17, 2014, page 2, first paragraph. This paragraph reads as follows:

"*The solution provided by the present invention is to target a region within exon 53 spanning the annealing sites H53A (+23+47) and/or H53A (+39+69) with a morpholino antisense oligonucleotide ('PMO') of 20-31 nucleotides in length.*"

From this statement it can be concluded that it is the intention of patentee to claim not only one of the two annealing sites (feature b_1) or b_2), respectively), but also the area from nucleotide +23 until +69. This is supported by Figure A which was also submitted on November 17, 2014 by patentee's representatives. This "area" is nowhere disclosed in the application as originally filed. Therefore, Article 123(2) EPC is violated.

According to feature a_2) the claimed oligonucleotide should be 20-31 nucleotides in length. Concerning the length of the oligonucleotides a paragraph on page 21, lines 9-17 of WO 2006/000057 can be found. This passage generally says that the antisense oligonucleotides can be as short as 12 bases whereas such length are not as efficient as longer (20-31 bases) oligonucleotides. The selection of 20-31 bases is therefore a selection of a list of various lengths of oligonucleotides. When we consider the relevant passage on page 62 of WO 2006/000057 there is no range of oligonucleotides provided. Table 39 summarizes 12 antisense oligonucleotides having different lengths. It should be noted that only two target regions are features of the claims, namely b_1) and b_2). The antisense oligonucleotide H53A (+23+47) has a length of 25 bases and the oligonucleotide H53A (+39+69) has a length of 31 bases. Those two embodiments corresponding to SEQ ID NO: 193 and 195 cannot form a basis for the range of 20-31 nucleotides.

It seems that the feature that the antisense oligonucleotide is a morpholino antisense oligonucleotide (feature c) of claim 1) has a potential basis on page 17, line 3 of the published international application. This passage relates, however, to Table 1A wherein all sequences designated as SEQ ID NOs: 1-211 are summarized. Alternatively in the relevant sentence on page 17, lines 2-4, other antisense chemistries such as peptide nucleic acids are, however, mentioned.

A basis for feature d), namely that the oligonucleotide induces exon 53 skipping might potentially be found on page 62 and in particular Table 39 of WO 2006/000057.

In Table 39 there are mentioned several antisense oligonucleotides whereby nine oligonucleotides are designated as H53A. The splice sites at the beginning and end of the exon are, however, different.

Patentee has selected from Table 39, which can be considered as a list, among nine individual oligonucleotides only two and the reason for selecting features b_1) and b_2), respectively, is not evident. When we look at the biological activity (ability to induce skipping) H53A (+39+69) corresponding to SEQ ID NO: 193 (feature b_2)) is said to induce strong skipping to 50 nM. The annealing site of feature b_1), namely H53A (+23+47) corresponding to SEQ ID NO: 195 is said to induce only very faint skipping to 50 nM.

According to claim 2 SEQ ID NO: 192 which corresponds to H53A (+36+62) should also fall under claim 1 and shows only a faint skipping at 50 nM (compare with Table 39).

Considering the other biological activities, namely the ability to induce skipping of other antisense oligonucleotides we noticed that for example H53A (+45+69) can induce faint skipping at 50 nM or H53A (+150+176) can also induce very faint skipping at 50 nM.

Therefore, features b_1) and b_2), respectively, were arbitrarily selected and it seems that the selected oligonucleotides do not fulfill the feature d) of claim 1, namely that the oligonucleotide induces exon 53 skipping since Table 39 shows varying degrees of biological activity down to very faint skipping.

3.1.2 Specifically hybridizable

When considering the term "specifically hybridizable to" in claim 1 it is more likely that also oligonucleotides that have little or faint skipping activities were used as basis. The description only provides a vague definition for "specifically hybridizable" in paragraphs [0052] and [0053], reading, e.g. *"a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target"* (see para. [0052]).

The specification as originally filed defines on page 23, lines 16-31 the term "specifically hybridizable". An antisense molecule is considered to be specifically hybridizable when binding of this compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility and there is a specific degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e. under physiological conditions in the case of in vivo assays or in the case of in vitro assays.

An oligonucleotide is therefore only "specifically hybridizable" when two conditions are fulfilled:

1) The antisense molecule must specifically hybridize with the target DNA or RNA and

2) Non-specific binding of the antisense compound to non-target sequences must be avoided. It is evident that this second condition requires that it had to be tested whether an oligonucleotide does bind or does not bind in a non-specific way to non-target sequences.

As potential basis for claim 1 pages 62-63 of the originally filed documents may serve. This section can, however, not serve as a basis for the feature "specifically hybridizing" for three reasons:

1) The strength of binding by hybridization and skipping is not the same;

2) Only the antisense oligonucleotide designated as H53A(+39+69) is said to induce strong skipping whereas the other oligonucleotides show only faint or very faint skipping speaking for a comparatively weak hybridization;

3) There is no disclosure whatsoever that those oligonucleotides do not specifically bind to non-target sequences.

Therefore, the passage in the general part of the specification on page 23 cannot be combined with the disclosure of Table 39. With other words: There is no disclosure that the antisense oligonucleotides disclosed on page 62 have the feature of "specifically hybridize to".

3.2 <u>Selection from at least two lists</u>

The opposed patent is to be revoked since granted claim 1 is an inadmissible selection from more than two lists of features which violates the requirements of Article 123(2) EPC (e.g. T223/11, T1651/11).

The combination of features created by patentee is not admissible under established jurisprudence of the Boards of Appeal considering the aspect that the features are selected from several lists. This is not allowable in view of the quoted decisions of the Board of Appeal. Of course there are many more T-decisions which could also be quoted in order to support this argument.

Furthermore, dependent sub-claims 2-9 are directly or indirectly referred back to claim 1. There is no disclosure wherein the features are originally disclosed in combination. Therefore, patentee has selected the features from different lists (e.g. former subclaims) which is a clear violation of the established case law of the Boards of Appeal.

The objected claims have therefore to be revoked, because the presently claimed combination of this feature is not disclosed and the claims are considered to be an artificially created new embodiment (e.g. T 2496/10, point 4.6).

IV. Article 100(b) EPC

Claim 1 relates to oligonucleotides with 20 to 31 nucleotides in length. It is submitted that the claimed invention is not enabled over the whole scope of the claim, in particular for oligonucleotides having 20 to 24 oligonucleotides.

The whole scope of the claim is not supported by the description, in view of the low predictability in the art, especially for the annealing range of +39+69, because the description provides data for only 191(+45+69),192(+39+62) and 193(+39+69) but no others.

These relevant oligomers from Table 39 have the length of 25, 24 and 31 base pairs respectively. In view of this, the description of the opposed patent fails to support whether oligonucleotides having the length of less than 24 base pairs actually have skipping activity. In this context, Corey and Abrams (Genome Biology 2001, 2 (5); 1015.1-1015.3, submitted as document **D1**) teach for morpholino antisense oligonucleotides that "this binding is no tighter than binding of analogous DNA and RNA oligomers, necessitating the use of relatively long 25-base morpholinos for antisense gene inhibition" (page 1015.1, left column, 4th to 2nd lines from the bottom).

In the absence of experimental data for oligonucleotides having the length of less than 24 base pairs, and in view of the teaching by Corey and Abrams (**D1**), it must be concluded that oligonucleotides having the length of less than 24 base pairs are not supported in the description.

Furthermore, it should be stressed that the only oligonucleotide which shows according to Table 39 a strong skipping ability has a length of 31 nucleotides. Contrary thereto shorter oligonucleotides like H53D(+14-07) having a length of 21 oligonucleotides or H53A(-12+10) having a length of 22 oligonucleotides or H53A(+07+26) having a length of 20 oligonucleotides

show only a very faint skipping or no skipping ability at all. Therefore, the opposed patent is to be revoked in view of lack of enabling disclosure.

V. Priority

The opposed patent claims the priority of the Australian patent application no. 2004903474 as filed on June 28, 2004. None of the claims is entitled to the priority of the Australian patent application. Claim 1 relates to two annealing sites (feature b_1) and feature b_2)) or both (feature b_3)). None of those annealing sites has a basis in the priority document.

The oligomers disclosed in the priority application are summarized in Table 1 in the specification of the priority document submitted as **D1**. Table 1 is shown in the following:

Brief Description of the Sequence listings

SEQ ID	SEQUENCE	NUCLEOTIDE SEQUENCE (5' - 3')
1	H8A(-06+18)	GAU AGG UGG UAU CAA CAU CUG UAA
2	H8A (-03+18)	GAU AGG UGG UAU CAA CAU CUG
3	H8A(-07+18)	GAU AGG UGG UAU CAA CAU CUG UAA G
4	H8A(-06+14)	GGU GGU AUC AAC AUC UGU AA
5	H8A(-10+10)	GUA UCA ACA UCU GUA AGC AC
6	H7A(+45+67)	UGC AUG UUC CAG UCG UUG UGU GG
7	H7A(+02+26)	CAC UAU UCC AGU CAA AUA GGU CUG G
8	H7D(+15-10)	AUU UAC CAA CCU UCA GGA UCG AGU A
9	H7A(-18+03)	GGC CUA AAA CAC AUA CAC AUA
10	C6A(-10+10)	CAU UUU UGA CCU ACA UGU GG
11	C6A(-14+06)	UUU GAC CUA CAU GUG GAA AG
12	C6A(-14+12)	UAC AUU UUU GAC CUA CAU GUG GAA AG

13	C6A(-13+09)	AUU UUU GAC CUA CAU GGG AAA G
14	CH6A(+69+91)	UAC GAG UUG AUU GUC GGA CCC AG
15	C6D(+12-13)	GUG GUC UCC UUA CCU AUG ACU GUG G
16	C6D(+06-11)	GGU CUC CUU ACC UAU GA
17	H6D(+04-21)	UGU CUC AGU AAU CUU CUU ACC UAU
18	H6D(+18-04)	UCU UAC CUA UGA CUA UGG AUG AGA
19	H4A(+13+32)	GCA UGA ACU CUU GUG GAU CC
20	H4D(+04-16)	CCA GGG UAC UAC UUA CAU UA
21	H4D(-24-44)	AUC GUG UGU CAC AGC AUC CAG

Table 1: Description of 2'-O-methyl phosphorothioate antisense oligonucleotides that have been used to date to study induced exon skipping during the processing of the dystrophin pre-mRNA.

Only skipping of exons 8, 7, 6 and 4 is attempted in the priority application.

Thus, exon 53 skipping and oligomers therefor, H53A (+23+47) and H53A (+39+69), are not disclosed in the priority application and the claims are therefore not entitled to the priority of June 28, 2005.

As the H53A (+23+47) and H53A (+39+69), as exemplary embodiments of claim 1, are not entitled to the priority, inventiveness of claims 1 to 9 is to be evaluated as of the international filing date of **28 June 2005**.

VI. Inventive Step

6.1 WO 2004/083432 (**D3**)

Claim 1 of the opposed patent relates to

a) an isolated antisense oligonucleotide that
   - a_1) binds to human dystrophin pre-mRNA,
   - a_2) wherein said oligonucleotide is 20 to 31 nucleotides in length and

b) is an oligonucleotide that is specifically hybridizable to
   - b_1) an exon 53 target region of the Dystrophin gene designated as annealing site H53A (+23+47),

b_2) annealing site H53A (+39+69), or

b_3) both

c) wherein said antisense oligonucleotide is a morpholino antisense oligonucleotide and

d) wherein said oligonucleotide induces exon 53 skipping.

D3 (WO 2004/083432) discloses an antisense oligomer named "h53AON1", which enables exon 53 skipping and has the following sequence (see Table 2 on page 48):

Table 2
Characteristics of the AONs used to study the targeted skipping of 15 different DMD exons*

Name	Antisense sequence (5'-3')	Length (bp)	G/C%	U/C%	Exon skip	Transcript
h53AON 1	cuguugccuccgguucug	18	61	72	+	OF

The complementary sequence of h53AON1 is CAGAACCGGAGGCAACAG. This sequence is completely encompassed in the claimed annealing sites b_2) and b_3) of the subject patent (see below where the yellow highlighted part of b_2) corresponds to the complementary sequence of h53AON1).

b_2): **CACCTTCAGAACCGGAGGCAACAGTTGAATG**

Therefore, **D3** discloses antisense oligonucleotide having feature b).

In addition, h53AON1 is shown to have a high skipping activity (see Figure 1E etc.). **D3** also discloses that the oligomers can be modified with morpholino phosphorodiamidate (see page 10, line 11 to page 11, line 4). Thus, the features c) and d), i.e. "wherein said antisense oligonucleotide is a morpholino antisense oligonucleotide, and, wherein said oligonucleotide induces exon 53 skipping", are disclosed in **D3**.

h53AON1 per se has a length of 18 oligonucleotides and does not satisfy requirement a_2) "wherein said oligonucleotide is 20 to 31 nucleotides in length".

However, **D3** states "*[c]urrently, many different compounds are available that mimic hybridization characteristics of oligonucleotides. Such a compound is also suitable for the present invention is such equivalent comprises similar hybridization characteristics in kind not necessarily in amount....As mentioned, oligonucleotides of the invention do not have to consist of only oligonucleotides that contributes to hybridization to the targeted exon. There may be additional material and/or nucleotides added.*" (see page 12, lines 11 to 20 of **D3**).

Based on the above statement, an average person of the skill in the art would have easily conceived of a "modification" of h53AON1 having additional nucleotides added, which may well be of the length of 20 to 31 nucleotides. Further, when two nucleotides are added to h53AON1 to make a 20mer equivalent, with any combination of those two nucleotides, at least 90% of the entire stretch of the 20mer equivalent is still completely identical to the counterpart region of the b_2) annealing site. Thus, it is clear that such 20mer is "specifically hybridizable to" b_2).

Considering the teaching by Corey and Abrams (**D1**) wherein a longer oligonucleotide is recommended for skipping (see supra), an average person skill in the art would have been motivated to modify h53AON1 by adding extra nucleotides to make it longer.

Therefore, the subject matter of claim 1 lacks inventive step over **D3** since **D3** contains the suggestion to elongate short nucleotides.

5.2 <u>WO 2004/048570 (**D4**) in view of **D3**</u>

The international patent application WO 2004/048570 was originally published in Japanese language on September 30, 2004. The document is therefore pre-published prior art. The English translation of this document was published as EP 1 568 769 and we refer in the following to this English document as **D4**.

D4 discloses oligomers enabling exon 53 skipping (see Examples 79-87), among which AO95 of Example 87 having SEQ ID NO: 75: corresponds to the 30th to 47th nucleotides of exon 53.

SEQD ID NO:75 of the sequence listing shows the nucleotide sequence of the oligonucleotide prepared in Example 87 (A095) (page 216, line 29).

<210> 75
<211> 18
<212> DNA
<213> synthetic oligonucleotide

<400> 75
ctgaaggtgt tcttgtac

The complementary sequence of AO95 is GTACAAGAACACCTTCAG.

This sequence is completely encompassed in the claimed annealing sites b_1) and b_3) of the subject patent (see below where the yellow highlighted part of b_1) corresponds to the complementary sequence of AO95).

b_1): **GGATGAAGTACAAGAACACCTTCAG**

Thus, AO95 is contained within SEQ195 (+23+47) of the subject claim. AO95 is demonstrated to have a skipping activity (see Figure 19 and para. [0319] of EP1568769A1). **D4** also discloses that the compound may be of morpholine salt (see para. [0046] of EP1568769A1).

AO95 per se is in the length of 18mer and does not comply with requirement a_2) "wherein said oligonucleotide is 20 to 31 nucleotides in length". However, **D3** states "*[c]urrently, many different compounds are available that mimic hybridization characteristics of oligonucleotides. Such a compound is also suitable for the present invention is such equivalent comprises similar hybridization characteristics in kind not necessarily in amount....As mentioned, oligonucleotides of the invention do not have to consist of only oligonucleotides that contributes to hybridization to the targeted exon. There may be additional material and/or nucleotides added.*" (see page 12, lines 11 to 20 of **D3**).

Based on the above statements of **D3**, a person skilled in the art would have easily conceived of a variant of AO95 having additional nucleotides added, which may well be of the length of 20-31. Further, when two nucleotides are added to AO95 to make a 20mer equivalent, with any combination of those two nucleotides, at least 90% of the entire stretch of the 20mer equivalent is still completely identical to the counterpart region of the b_1) annealing site. Thus, it is clear that the 20mer equivalent "specifically hybridizable to" b_1).

A person skilled in the art starting from the specific disclosure as explained above in document **D3** or **D4**, respectively, and looking for an alternative embodiment has a strong motivation to elongate the 18mers as disclosed in **D3** or **D4**, respectively, by at least 2-10 oligonucleotides since it can be expected that the binding of the oligonucleotide will be improved by such an elongation as taught by **D3** or **D1**, respectively.

Therefore, the claimed invention is rendered obvious for the average person skilled in the art by either a combination of **D3** and **D4,** or a combination of **D3** and **D1**.

5.3 <u>The subject matter of claim 1 does not exert superior effect in full scope.</u>

"h53AON1" has been cited as a prior art by the Examining Division of EPO in the examination stage of the subject patent. In the opinion attached to the extended European search report dated January 2, 2013 the searching authority cited inter alia documents D1, D2 and D3. These documents are submitted as document **D5** (CA 2 507 125), **D6** (Human Molecular Genetics, vol. 12 (2003), pages 907-914) and **D7** (Neuromuscular Disorders, vol. 12, January 2002, pages S71-S77). In section 3 the searching authority concluded that all of documents D1-D3 (now corresponding to **D5-D7**) disclose antisense oligonucleotides targeting exon 53 in the region corresponding to H53A (+23+47) and H53A (+39+69) for inducing exon skipping in the dystrophin gene in order to treat DMD.

Regarding "h53AON1", the University of Western Australia argued that H53A (+39+69) is superior over h53AON1 in terms of skipping activity (see Annex 2 - response filed by the representatives of the University of Western Australia dated 5 November 2013).

Nippon Shinyaku (NS) conducted experiments to see whether any oligomers falling within the scope of claim 1 of the subject patent have superior activity over h53AON1 and found that some oligomers have inferior activities to h53AON1. The experimental report was performed by opponent and is submitted as document **D 8**.

The data as shown in the experimental report submitted as **D8**, confirm that the alleged superior activity as argued by the patentee in the examination stage is not obtainable over the whole scope of the claim, and thus the claimed subject matter is not inventive.

Since no superior effect has been shown over the closest prior art the object of the opposed patent is to provide an alternative oligonucleotide. Such an alternative is, however, rendered obvious by the prior art, in particular by a combination of **D3** and **D1** or **D4** and **D3**.

The claimed subject matter is not inventive in view of the relevant prior art.

Conclusion

Since the opposed patent violates the European patent convention in several respect, the complete revocation of the opposed patent is justified.

Dr. G. Keller

Enc.:
1 copy
payment order
D1-D8, 2-fold

LEDERER & KELLER
Patentanwälte Partnerschaft mbB
European Patent and Trademark Attorneys

EPO - Munich
100
29. Sep. 2017

DR. A. VAN DER WERTH
(1934 – 1974)
DR. FRANZ LEDERER
(1963 – 2012)
DR. GÜNTER KELLER
Dipl.-Biol.
DR. MICHAEL BEST
Dipl.-Chem.
DR. STEPHAN TEIPEL
Dipl.-Chem.
DR. GEORG KALHAMMER
Dipl.-Biochem.

80538 MÜNCHEN
Unsöldstraße 2
Telefon +49 89 21 23 99 0
Telefax +49 89 21 23 99 22
E-Mail info@lederer-keller.de

European Patent Office
Postbus 5818

2280 HV Rijswijk

Netherlands

Your reference

Our reference
ESP00349NIP

29 September 2017
K/sh

Re.: European Patent No. 2 206 781 B1
European Patent Application No. 10004274.6
Patentee: The University of Western Australia
Opponent: Nippon Shinyaku Co., Ltd.

This is in response to the summons to attend oral proceedings pursuant to Rule 115(1) EPC dated March 30, 2017.

I. Main Request

1.1 Article 123(2) EPC

The Opposition Division expressed its preliminary opinion that the Main Request extends beyond the disclosure of the application as originally filed (in respect of the binding of both sites, the morpholinos and the combination of morpholinos with any given oligonucleotide targeting the two sites H53A(+23+47) and H53A(+39+69)). Opponent agrees with this preliminary opinion. In the following we would like to comment the arguments of patentee submitted on February 22, 2017:

1.1.1 Intermediate generalization

Claim 1 comprises several features of the isolated antisense oligonucleotide which do either not have any literal support or are picked from several different passages of the original specification whereby it is not disclosed that all features relate to a preferred embodiment.

1.1.1.1 Three annealing sites

Claim 1 comprises three annealing sites whereby feature b_3 relates to "both" annealing sites, namely H53A(+23+47) and H53A(+39+69). Patentee was not able to refer to a literal disclosure but argued that it is allegedly not required to have a strict literal basis for the term "both" provided such feature can be directly and unambiguously derived from the content of the application as filed.

We strongly disagree with this argument since none of the passages quoted by the patentee may serve as a direct and unambiguous support for the feature "both".

a) Table 39

Patentee refers to Table 39 on pages 62 and 63 of the specification as filed and alleges that a person skilled in the art would, on viewing the application and the data as a whole, directly and unambiguously understand that the target annealing site encompasses both, namely H53A(+23+47) and H53A(+39+69). Patentee does, however, not explain the reason <u>why</u> the person skilled in the art should understand that <u>both</u> annealing sites should be suitable. In Table 39 there is only one AON which is disclosed to have a strong skipping activity, namely H53A(+39+69). Furthermore, there are two AONs, namely H53A(+45+69) and H53A(+39+62) which are disclosed to have a faint skipping activity. Those two AONs are located in the same annealing site (HA53A(+39+69)).

Furthermore, there are three AONs disclosed which are said to have very faint skipping activity, namely H53A(+23+47) which corresponds to the first annealing site and two AONs which are outside of both annealing sites, namely H53D(+14-07) and H53A(+150+176). From the "very faint skipping" ability the person skilled in the art may speculate that there are at least three other regions which may potentially be interesting. The person skilled in the art learns on the other hand from Table 39 that variations in the oligonucleotide positions of the AONs may easily lead to a complete loss of skipping activity since several (4) of the tested AONs did not show any skipping activity at all.

Patentee's argument that four out of seven AONs in Table 39 skipped exon 53 is not convincing since there are also three other AONs which showed a faint skipping and which are located outside of the claimed annealing site. Since the ability to induce skipping varies substantially from strong skipping to very faint skipping and since very faint skipping is reported to occur also at other annealing sites, e.g. H53D(+14-07) and H53A(+150+176), the

person skilled in the art cannot deduce unambiguously and directly from this Table that both annealing sites of claim 1 are suitable to perform exon skipping. To the contrary it is not possible to conclude from the data of Table 39 directly and unambiguously that there is a binding region which may be directly and unambiguously derived from the AONs of Table 39.

b) Page 36, lines 19-20

Patentee referred to page 36, lines 19-20 of the application as filed wherein is said that once efficient exon skipping had been induced with one antisense molecule, subsequent overlapping antisense molecules may be synthesized and then evaluated in the assay as described above.

This sentence may be understood as an invitation to perform a research programme and to synthesize and study several different AONs and to check whether skipping occurs or not. This is, however, not a clear and unambiguous disclosure of a combination of both annealing sites as claimed in claim 1 of the Main Request. It should also be mentioned that cited T 0667/08 does not support patentee's position. This decision states in section 4.1.4:

"*... it is therefore essential, when deciding on issues of added subject-matter, to identify the actual teaching conveyed by the original disclosure, i.e. the technical information that the skilled person reading the original disclosure would have derived from its content (description, claims, drawings) considered in its entirety.*

This approach might lead to the identification of subject-matter which has not been explicitly revealed as such in the application as filed, but nevertheless derives directly and unambiguously from its content. Literal support is not required by the wording of Article 123(2) EPC. An amendment can therefore be allowable if it combines information which has not been disclosed in one and the same section of the original disclosure, but results, for instance, from information gathered from various embodiments possibly associated with general statements regarding the information derivable from the introductory section of the application."

The information which is objectively derivable from Table 39 does not lead the person skilled in the art directly and unambiguously to the feature b_3 (both) of claim 1. Therefore, we believe that the Opposition Division is perfectly correct in its preliminary opinion that the term "both" is not directly and unambiguously derivable from the content of the application as originally filed.

1.1.1.2 Nucleotides in length

As basis for the length of the nucleotides patentee referred to page 21, second paragraph of WO 2006/000057. It seems that this is the only passage where different lengths of the antisense oligonucleotides are disclosed. The sentence starting on page 21, line 12 refers to targets such as exon 19 and it continues to disclose that short oligonucleotides such as 12 bases were able to induce exon skipping which were, however, not as effective as longer oligonucleotides (20-31 bases). In the second sentence it is disclosed that in some other targets antisense oligonucleotides having 23, only 17 or 25 nucleotides were disclosed. The quoted sentences teach the person skilled in the art that for the different exons various lengths of the antisense oligonucleotides were used. Interestingly, however, in this passage exon 53 is not even mentioned. Therefore, patentee has selected from a list of different lengths of oligonucleotides, namely 12 bases, 20-31 bases, 17 oligonucleotides and 25 oligonucleotides one range and combined it with an exon which is not even mentioned in this paragraph. Patentee has therefore selected from a list of about 50 exons one exon and has combined this with one length of oligonucleotides out of four different options although the selected length of the oligonucleotides was disclosed in connection with exon 19 which is not subject of claim 1.

It is more than evident that patentee has selected features from two passages (lists) of the original disclosure and combined them although originally no relationship between the two features was disclosed.

1.1.1.3 Morpholino

It is true that on page 17, line 3 morpholinos are mentioned. The quoted sentence makes, however, clear that for certain antisense chemistries such as peptide nucleic acids or morpholinos the uracil (U) bases may be shown as thymidine (T).

It should, however, be kept in mind that the opposed patent describes very detailed the modification of the oligonucleotides. In order to avoid degradation of pre-mRNA during duplex formation with the antisense molecules the antisense molecules used in the method of the opposed patent may be adapted to minimize or prevent cleavage by endogenous RNase H (page 25, lines 15-17 of the application as originally filed).

There are many different modifications disclosed in the passage starting on page 25, line 15 and ending on page 28, line 6 of WO 2006/000057. To select only the morpholinos and to combine this feature with the target regions (features b) of claim 1) is a further selection from a further different list.

1.1.1.4 Inducing exon skipping

Patentee referred to Table 39 on pages 62-63 of the application as originally filed. It should be noted, however, that this Table does not directly point to a binding region of exon 53 since there are several antisense oligonucleotides disclosed which are directed to exon 53 but do not induce skipping, namely H53A(-12+10), H53A(-07+18), H53A(+07+26) and H53A(+124+145). Those results have simply not been mentioned by patentee.

It should be kept in mind that the patentee has made an inadmissible selection from at least four lists, namely

a) the exon,
b) the modification of the oligonucleotide,
c) the length of the oligonucleotides in claim 1, and
d) the annealing sites.

1.1.1.5 Specifically hybridizable

The relevant passage wherein the term "specifically hybridizable" is disclosed in the application as originally filed (page 23, lines 16-31) has been cited correctly by patentee on page 4 of the last submission. Our argument with regard to Article 123(2) EPC is that this passage is according to the original disclosure related to each and every oligonucleotide as disclosed in the application of the opposed patent. It is, however, not related to oligonucleotides which bind to a particular annealing site in exon 53 and which have a specific length.

The inadmissible broadening is more evident when this passage is read in combination with the disclosure of Table 39. The quoted passage on page 23, lines 16-31 of the original disclosure broadens the claim by allowing oligonucleotide substitutions so that the oligonucleotide must not be 100% complementary to the target sequence whereby only unspecific binding should be avoided.

To combine this passage with the disclosure of Table 39 may have the consequence that the sequence of the provided antisense oligonucleotides may be changed by nucleotide substitutions. The person skilled in the art will, however, learn from Table 39 wherein only one oligonucleotide shows a strong skipping ability and all other oligonucleotides show only a faint, very faint or no skipping ability that it is very likely that the skipping ability will be dramatically reduced if changes of the oligonucleotide sequence are performed. There is no reason for the skilled artisan to believe that modifications of the AONs might have the ability to include skipping.

1.1.1.6 Selection from two lists

Patentee referred to T 783/09 in order to argue that the features of opposed claim 1 are not selected from two lists in an inadmissible manner. In T 783/09 the Board decided that a claim comprising only three out of 44 combinations derivable from two lists was allowable. The factual situation of T 783/09 is, however, completely different from the present case since a selection was made from several different lists such as number of exon, length of oligonucleotides, target site, selectively hybridizable and modification of the oligonucleotide in order to obtain the combination of features of claim 1. The number of combinations derivable from the lists amounts to several thousands of potential combinations. Therefore, T 783/09 is not applicable for the present case.

1.1.2 Dependent claims

It is true that we did up to now not analyze in detail the features of claims 2 to 9. The reason therefore is that all claims are directly or indirectly dependent from claim 1. If claim 1 has to be revoked due to violation of Article 123(2) EPC the dependent claims will share this fate. In case the objections of claim 1 can be overcome we reserve the right to present the then relevant arguments with regard to the features of the claims dependent from claim 1.

As an intermediate result it can be stated that claim 1 has to be revoked due to a violation of Article 123(2) EPC for several reasons and the claims dependent from claim 1 share this fate.

1.2 Article 100(b)/83 EPC

In its reply (page 8) patentee has argued that the burden of proof for non-enablement lies with the opponent and that the opponent has filed no data to support the allegation that AONs of 20 to 24 nucleotides would not work. We do not agree with patentee since we have presented serious doubts, substantiated by verifiable facts.

1.2.1 Evidence which causes serious doubts

1.2.1.1 (D1)

We have already submitted document D1 which has been published before the priority date of the opposed patent. It is true that the morpholino antisense oligonucleotides are described as tools for investigating vertebrate development. On the other hand the molecular mechanisms are based on molecular principles which are in general applicable in vertebrate organisms. Therefore, the statement of D1 that relatively long oligomers (morpholinos) of at least 25 nucleotides are required for exon skipping is valid.

1.2.1.2 (D9)

Document D9 was cited by patentee and published after the filing date of the opposed patent confirms the finding of D1. On page 108, right column, lines 1-9 from the bottom, the following sentence can be found:

"*Indeed, the fact that the 30 mer PMOs (-G,-H) were more bioactive than the 25 mer PMO counterpart (-A) targeted to the same open/accessible sites of the exon would suggest that strength of binding of PMO to the target site may be the most important factor in determining PMO bioactivity.* ***The influence of AO length on bioactivity has been reported elsewhere [9,30], and is further confirmed in the present study; all 30mers tested were more bioactive relative to their 25mer counterpart.***" (emphasis added)

The article continues on page 109, left column:

"*The reason that our PMOs produce higher levels of exon skipping could be a (combined) consequence of the different AO chemistries, length of AO used, type of cell used (patient vs. control) and the absolute target site of AO.*"

Consequently the literature teaches the person skilled in the art that short oligonucleotides of 20 to 23 oligonucleotides will have no skipping activity. The opposed patent does not contain an example showing the contrary.

From the data provided in Table 1 and Figure 1 it can be learned that the 30 mer PMO-G and PMO-H produce higher levels of skipping relative to the 25 mer PMO-A and PMO-B. Consequently a further reduction of skipping activity can be expected when the AOs are further shortened.

1.2.1.3 (D8)

Opponent has also provided evidence (D8) showing that short antisense oligomers having a length of 18 to 21 nucleotides do not induce sufficiently exon 53 skipping. Therefore, there are serious doubts which have been substantiated by verifiable facts that the claimed invention cannot be worked over the whole claimed range, in particular when short AONs are used.

1.2.2 "Specifically hybridizable"

Claim 1 is substantially broadened by the feature "specifically hybridizable to an exon 53 target region" which means that the claimed antisense oligonucleotide must not match exactly to the exon 53 target region, but also such AONs are encompassed which must only have a sufficient degree of complementarity that a stable and specific binding occurs between the oligonucleotide and the DNA or RNA target.

By using the feature "specifically hybridizable" the claimed oligonucleotide must not be 100% complementary to the target sequence ([0053], first sentence). Therefore, at least one or even more nucleotides can be replaced by other nucleotides which do not perfectly match with the target sequence. The consequence of lowering the complementarity to a value below 100% is necessarily that the skipping ability will be reduced. When very faint skipping ability is further reduced, because the complementarity of antisense molecule and target sequence is below 100% the skipping ability will not be only faint but also hardly be detectable.

Patentee has not provided any evidence that an oligonucleotide which is not 100% complementary to the target sequence shows any skipping activity. In view of the serious doubts which are substantiated above the burden of proof that the requirements of Art. 83 EPC are met rests on patentee.

Since opponent has provided evidence which causes serious doubts substantiated by verifiable facts that the claimed Invention cannot be worked over the whole claimed range, the burden of proof rests with patentee to produce sufficient evidence that the claimed invention is disclosed in an enabling manner over the whole scope of the claims, in particular with regard to comparatively short oligonucleotides of 20 mer to 25 nucleotides which are not 100% complementary to the target sequence.

1.3 Article 56 EPC (inventive step)

The arguments of patentee relating to inventive step can be found in sections 3.0 (Background to the Invention, pages 6, 7) and 6.0 (Inventive Step, pages 9-14).

1.3.1 Post-published evidence (D9)

It seems that patentee tries to transfer disclosure from the post-published document D9 into the disclosure of the present patent in order to bolster the argumentation relating to inventive step. In general the disclosure of post-published documents can only be taken into account for the question of sufficiency of disclosure if it was used to back up the positive findings in relation to the disclosure of a patent application (T 1273/09 citing T 609/02).

It is, however, not admissible to combine the teaching of an application with the teaching of post-published evidence in order to support the presence of an inventive step or to transfer a statement from post-published evidence (D9) into the disclosure of the opposed patent as suggested on page 7 of applicant's submission dated February 22, 2017.

Contrary to patentee's allegation the opposed patent fails to state that targeting the claimed sequence between nucleotides 23 and 69 or between 30 and 65, respectively, results in skipping of exon 53.

Two arguments strongly contradict patentee's assertion:

First, the experimental section of the present specification reports that some antisense oligonucleotides have only a faint or very faint ability to induce skipping.

Second, D9 notes that the levels of exon skipping that may be considered effective is over 50% exon skipping (compare with page 108, right column, lines 14-17). Consequently D9 also

discloses that among 13 PMOs tested, only 6 PMOs, e.g. G, H, A, I, B and M show activities over 50% whereas other PMOs show lower activities. For example D9 describes that PMO-J has the activity of only 37% and that all other PMOs tested gave exon skipping at levels of between 15% and 26% (see page 104, right column, lines 12-14). That means that 6 out of 13 PMOs tested which is more than half of those tested show activities less than 50%, more precisely less than 40%. In addition D9 reports that even the PMOs with high skipping activity at one concentration also show the skipping activity as low as 30% at lower concentrations of e.g. 25 µM (see page 104, right column, lines 24-32). For the sake of completeness we would like to mention that the concentrations used in our experimental report D12 are 3, 10 and 30 µM.

Document D9 can therefore not support the allegation that the whole claimed targeting area is suitable.

It has furthermore been alleged that the authors of D9 who belong to the same research group as the present inventors have confirmed that the claimed target region is an advantageous target for exon 53 skipping. It should be considered that the authors of D9 belong to the same research group as the inventors of the present application. Therefore, the persons are either identical or at least well connected in a research network. It is also not proven that the disclosure of the present application inspired any skilled person outside the group of the inventors of the present patent.

A careful analysis of document D9 makes plainly evident that the conclusions drawn by patentee's representatives are not supported by the experimental evidence published in D9. Therefore, D9 is not relevant for two reasons, namely first, it is in general not admissible to use the post-published document as support for the presence of an inventive step and second, even if it was considered to be admissible the arguments of patentee are not supported by the facts published in D9.

1.3.2 Length and chemical backbone of oligonucleotide

Under section 6.1 (page 9) patentee stressed the fact that the oligonucleotide h53AON1 is outside of granted claim 1 due to (a) its length and (b) its chemical backbone. Patentee argued that the 18-mer AON of D3 fails to comply with the claimed requirements of being 20-31-mer and being PMO.

The Opposition Division correctly considered document D3 as the closest prior art. Uncontestably D3 discloses the oligonucleotide h53AON (page 48) having a length of 18 nucleotides. Document D3 is, however, not restricted to the disclosure of these single oligonucleotides in the examples. Claim 3 mentions that the oligonucleotide may have 14 to 50 nucleotides. In claim 17, 16 to 80 nucleotides are mentioned and on page 9, lines 27-30 it is disclosed that the oligonucleotides have preferably a length between 16 and 50 nucleotides. Therefore, D3 provides for the person skilled in the art the suggestion to use oligonucleotides which are longer.

D3 discloses also that the oligonucleotide may be a morpholino derivative. In this respect we refer to claim 12 and page 10, lines 14-16.

We agree therefore with the conclusion of the Examining Division regarding D3. If D3 is considered to be the closest prior art, the claimed subject matter is rendered obvious by D3 itself since D3 does not only disclose the highly relevant oligonucleotide h53AON but also that longer oligonucleotides are preferred and that the morpholino modification is preferred.

Furthermore, patentee argued (page 10) that D3 states that there may be additional nucleotides added to the 18-mer whereby, however, such additional nucleotides to be added do not contribute to hybridization by referring to the following statements of D3:

> *As mentioned, oligonucleotides of the invention <u>do not have to consist of only oligonucleotides that contribute to hybridisation</u> <u>to the targeted exon. There may be additional material and/or nucleotides added</u>.* [emphasis added]

First, patentee's argument is based on a misleading interpretation of the quoted sentence. The sentence says that in general added nucleotides may contribute to hybridization. In the exception there may also be additional nucleotides which do not contribute to hybridization. Regularly the oligonucleotides consist only of nucleotides which contribute to hybridization and are therefore complementary to the target sequence.

Second, we would like to submit that even if we followed patentee's interpretation for the sake of argument, the quoted sentence does not contradict our position. When the 18-mer of D3 is extended to 20-mer by adding two extra nucleotides, the 20-mer would have at least 90% sequence identity to the target sequence regardless of the base type of the two extra nucleotides since at least 18 out of 20 nucleotides would perfectly match the target sequence, such oligonucleotides might be considered as "specifically hybridizable".

Patentee's argument must be understood with the definition of "specifically hybridizable" provided in the present specification. The present specification states that "[i]t is understood in the art that the sequence of an antisense molecule need not be 100% complementary to that of its target sequence to be specifically hybridisable." (see paragraph 0053).

Patentee's counterargument is therefore not convincing. If the quoted passage is interpreted in a literal sense, the additional nucleotides are complementary to the target sequence. If, however, we follow for the sake of argument patentee's argument, namely that the additional nucleotides are not complementary to the target sequence, such a sequence would nevertheless fall under the claim since the claim does not require a 100% complementary in view of the feature "specifically hybridizable". Therefore, patentee's counterargument is groundless.

1.3.3 Does the claimed subject matter show a superior effect over the whole breadth of the claim?

1.3.3.1 D12 (patentee's experiments)

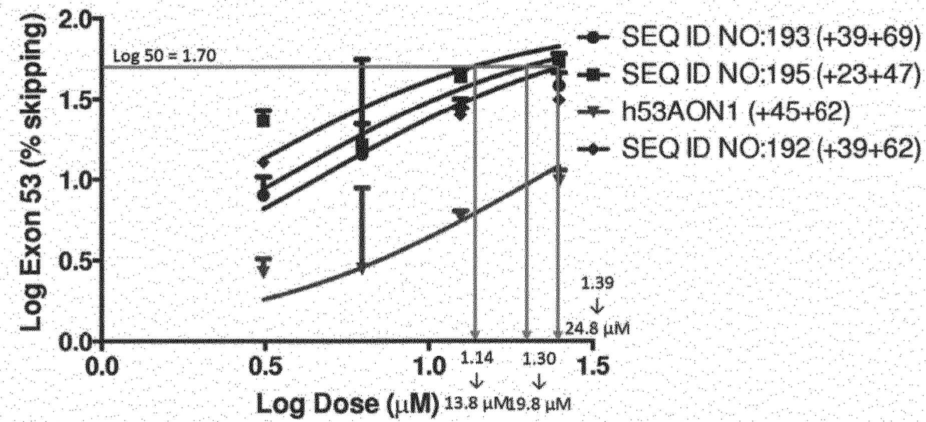
The experimental data of D12 as submitted by the patentee shall allegedly demonstrate that the AON of the opposed patent has higher activity than the 18-mer of D3. We do not agree.

The OD has acknowledged that AONs tested in D12 are of 24 to 31-mer sizes, and that whether smaller AONs of 20 to 21-mer sizes would have higher activities than those of 18-mer size cannot be appreciated from the results of D12. We agree to the OD's opinions on this point since there is no evidence on file to support that 20-23 mer oligonucleotides are more efficient than the prior art oligonucleotides or even equivalents.

Furthermore, the results as presented in D12 are not correct since the numerical values indicated as EC50 seem to have been incorrectly calculated.

RD Cells (page 2 of D12)

	EC50	
SEQ ID NO:193 (+39+69)	4.536	24.8
SEQ ID NO:195 (+23+47)	2.431	13.8
h53AON1 (+45+62)	21.25	-
SEQ ID NO:192 (+39+62)	3.531	19.0



The Opponent has calculated as schematically shown above the correct EC50 values for the RD cell data of D12, which are shown in the table below.

Correct EC50 values for RD cells

	(1) EC50 shown in D12(=EC10) (μM)	(2) Correct EC50 (μM)	Fold (=(2)/(1))
+39+69	4.536	**24.8**	5.47
+23+47	2.431	**13.8**	5.68
+39+62	3.531	**19**	5.38

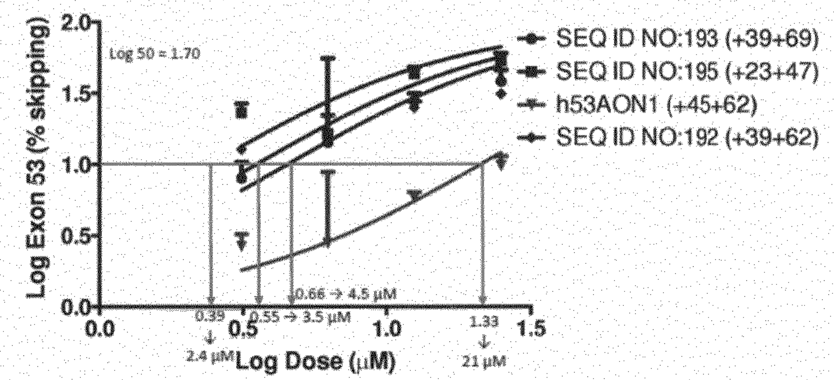
<u>The EC50 value for h53AON1(+45+62) was not available as the skipping activity was below 50%</u>. For the same reasons, the EC50 values for any of the AONs in myoblast were not available either.

The numerical values indicated in D12 as "EC50" are in fact those for EC10. EC10 can be obtained at intersection points of the horizontal line at 1.0 (=log10) of the vertical axis, i.e. "Log Exon 53 (% skipping)", and the curve for each sample.

The “EC50” value for SEQ ID NO 193 in RD cells as indicated in the table of D12 is 4.536. The common logarithm of 4.536 (=log4.536) equals to 0.6567. This is exactly the value of the intersection between the line at 1.0 of the vertical axis (=EC10) and the curve for SEQ ID NO:193.

RD Cells

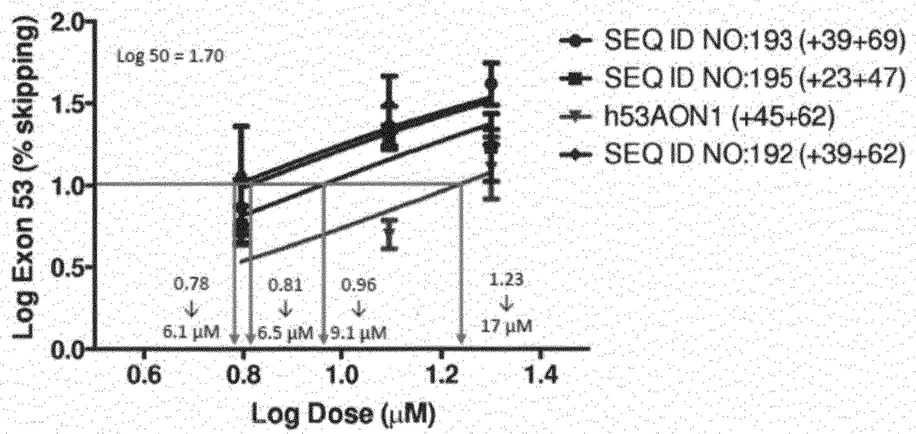
	EC50
SEQ ID NO:193 (+39+69)	4.536
SEQ ID NO:195 (+23+47)	2.431
h53AON1 (+45+62)	21.25
SEQ ID NO:192 (+39+62)	3.531



In analogous manner the values were recalculated for the experiments performed with myoblasts:

Myoblasts

	EC50
SEQ ID NO:193 (+39+69)	6.482
SEQ ID NO:195 (+23+47)	9.135
h53AON1 (+45+62)	17.13
SEQ ID NO:192 (+39+62)	6.061



Therefore, Patentee's arguments based on D12 cannot support the presence of an inventive step since the calculations are incorrect.

1.3.3.2 (D8)

Patentee criticizes in the submission of February 22, 2017 on page 13 that opponent has deliberately selected two sequences which significantly overlap and allegedly anneal to very similar regions within the annealing sites. It should be mentioned, however, that this is no valid counterargument since patentee has the burden to show that an alleged superior effect is obtainable over the whole scope of the claim (T 939/92).

It is true that opponent filed experimental evidence as report D8 using AONs targeting +45+62, +49+69 and +50+69. The sequences tested, in particular +49+69 and +50+69, are similar.

In addition, in the results D8 are criticized since the activities of the same AON change from the best to worst depending on the concentration.

Against this allegation, the Opponent wishes to point out that patentees allegation is in the same manner applicable to the data of D12. In the results of myoblasts, SEQ ID NO:192 (+39+62) achieves the highest exon skipping at the concentration of 0.8 (= 6.3 μM) whereas SEQ ID NO:193 (+39+69) achieves the highest at the concentration of 1.3 (=20 μM). In addition, in the result of RD cells of D12, SEQ ID NO:192 (+39+62) achieves the second highest exon skipping at the concentration of 0.5 (= 3 μM) whereas it achieves the third highest at 1.4 (= 24 μM).

Furthermore, it has been objected that controls are not present in D8. It seems, however, that also D12 does not contain controls.

In view of this objection opponent hereby submits an amended version of D8, i.e. D8-1, where the control of AON targeting the 39th to 69th nucleotides in exon 53 designated as "SEQ ID NO: 4" is added. In comparison to the control, the faintness of the skipping activities for SEQ ID NOs: 2 and 3 is further highlighted. In addition, the data for 3μM is deleted in D8-1, because it turned out that a concentration of 3 μM as presented in the original measurements (D8) was too low.

In addition, Opponent hereby submits another experimental report as D13. In D13, an extra set of AONs encompassed by claim 1 of the present patent is tested, namely SEQ ID NOs: 5 to 10. SEQ ID NOs: 1 and 4 are identical to those of D8-1, which are h53AON1 and +39+69, respectively. As can be seen in the table in "Results" of D13, it is clearly demonstrated that all of SEQ ID NOs: 5 to 10 have very faint skipping activities. A note should be made that the skipping activities for SEQ ID NOs: 5 to 10 do not escalate in dose dependent manners. Rather the skipping activities for SEQ ID NOs: 5 to 10 stays near 2.0% to 3.0% regardless of the doses.

At least based on D8-1, a person of skill in the art would undoubtedly understand that the patented invention fails to provide its intended effect over the entire scope of the claim. When considering the results shown in D13, said understanding will further be confirmed.

1.3.4 Summary regarding Art. 56 EPC

Regarding Article 56 EPC we agree with the preliminary non-binding finding of the Opposition Division that the claimed subject matter is not based on an inventive step.

II. Auxiliary Request 1

The Auxiliary Request 1 differs from the Main Request insofar that granted claims 4 and 5 are deleted. We agree with the preliminary opinion of the Opposition Division that Auxiliary Request 1 is not admissible in view of a violation of Rule 80.

Since claim 1 of Auxiliary Request 1 corresponds exactly to claim 1 of the Main Request our objections as raised for the Main Request apply also to Auxiliary Request 1.

III. Auxiliary Request 2

In claim 1 of the Auxiliary Request 2 the term "or both" has been deleted and the two annealing sites are connected by the conjunction "or".

Our objections as raised with regard to the Main Request apply also to Auxiliary Request 2 with the proviso that the objections relating to Article 123(2) EPC concerning "both" are not maintained for Auxiliary Request 2.

IV. Auxiliary Request 3

The objections as raised with regard to the Main Request are maintained with the proviso that specific objections relating to the annealing site H53A(+23+47) and to "or both" are not maintained.

V. Auxiliary Request 4

This seems to be a combination of the amendments proposed in Auxiliary Request 1 and Auxiliary Request 2.

In claim 1 of Auxiliary Request 4 the term "or both" has been deleted and the two annealing sites are connected by the conjunction "or".

The Auxiliary Request 4 differs from the Main Request insofar that granted claims 4 and 5 are deleted. We agree with the preliminary opinion of the Opposition Division that Auxiliary Request 4 is not admissible in view of a violation of Rule 80.

Since claim 1 of Auxiliary Request 4 corresponds exactly to claim 1 of the Auxiliary Request 2 our objections as raised for the Main Request apply also to Auxiliary Request 4 whereby our objections concerning "both" are no longer applicable.

VI. Auxiliary Request 5

This seems to be a combination of the proposed amendments as presented for Auxiliary Requests 1 and 3.

The objections as raised with regard to the Main Request are maintained with the proviso that specific objections relating to the annealing site H53A(+23+47) and to "or both" are not maintained.

Auxiliary Request 5 further differs from the Main Request insofar that granted claims 4 and 5 are deleted. We agree with the preliminary opinion of the Opposition Division that Auxiliary Request 5 is not admissible in view of a violation of Rule 80.

VII. Conclusion

Since there is no set of claims on file which complies with the requirements of the EPC the patent has to be revoked.

Dr. G. Keller

Enc.:
1 copy
D 8-1, 2-fold
D 13, 2-fold

EXHIBIT AV

REDACTED

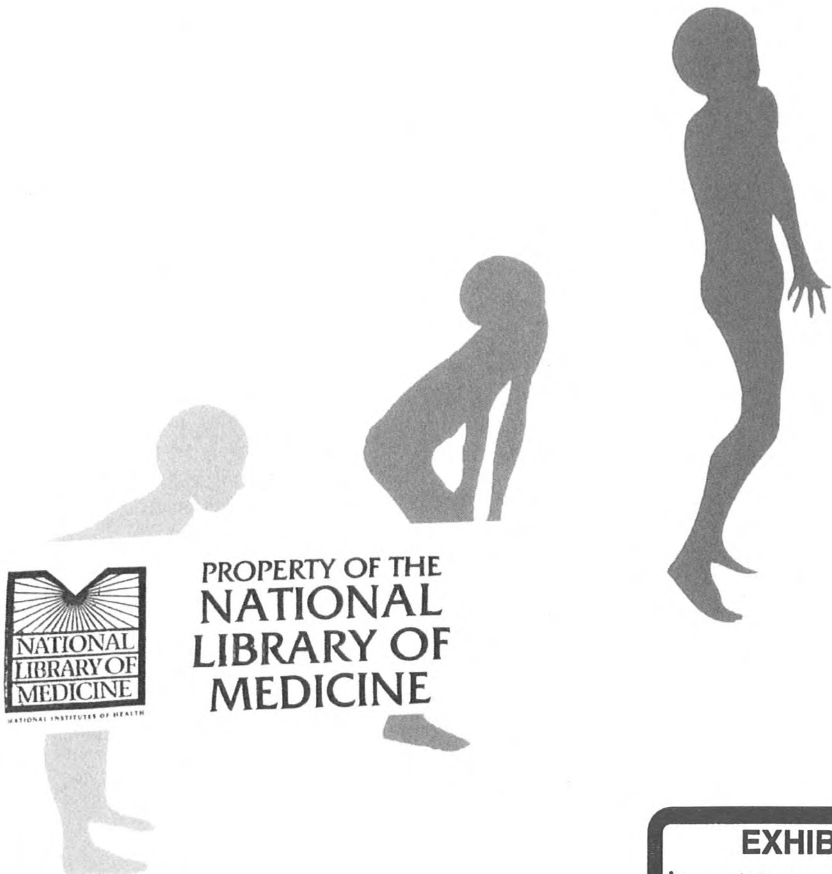
IN ITS

ENTIRETY

EXHIBIT AW

20 (2) 95–154
ISSN 0960-8966

Neuromuscular Disorders



ELSEVIER

Official Journal of the World Muscle Society

Neuromuscular Disorders

Aims and Scope

This international, **multidisciplinary** journal covers all aspects of neuromuscular disorders in childhood and adult life (including the muscular dystrophies, spinal muscular atrophies, hereditary neuropathies, congenital myopathies, myasthenias, myotonic syndromes, metabolic myopathies and inflammatory myopathies).

The Editors welcome original articles from all areas of the field:

- **Clinical aspects,** such as new clinical entities, case studies of interest, treatment, management and rehabilitation (including biomechanics, orthotic design and surgery);
- **Basic scientific studies** of relevance to the clinical syndromes, including advances in the fields of molecular biology and genetics;
- Studies of **animal models** relevant to the human diseases.

The journal is aimed at a **wide range** of clinicians, pathologists, associated paramedical professionals and clinical and basic scientists with an interest in the study of neuromuscular disorders.

In addition to original **research papers**; the journal also publishes **reviews** and mini-reviews, preliminary short communications and book reviews, and has editorial, correspondence and news sections. **Reports** on congresses and workshops, taking the form of a digested or very comprehensive commentary, pointing out some of the particular highlights in relation to the contributions and giving some detail of the area covered, important contributions and a list of participants, are also welcome.

The journal is published twelve times a year and aims at rapid publication of high-quality papers of scientific merit and general interest to a wide readership. There is also a fast track for rapid publication of new material of outstanding scientific merit and importance.

Printed by Polestar Wheatons Ltd, Exeter, UK

Neuromuscular Disorders

Editor-in-Chief
V Dubowitz

Volume 20 No. 2 (2010)

ELSEVIER
Amsterdam - London - New York - Oxford - Paris - Shannon - Tokyo

Author enquiries
For enquiries relating to the submission of articles (including electronic submission where available) please visit this journal's homepage at http://www.elsevier.com/locate/nmd. You can track accepted articles at http://www.elsevier.com/trackarticle and set up e-mail alerts to inform you of when an article's status has changed. Also accessible from here is information on copyright, frequently asked questions and more.
Contact details for questions arising after acceptance of an article, especially those relating to proofs, will be provided by the publisher.
Neuromuscular Disorders **proceeds entirely online at www.ees.elsevier.com/nmd** There is an online tutorial for authors and online support for technical problems or questions via Elsevier's AuthorSupport team (authorsupport@elsevier.com).

Publication information: *Neuromuscular Disorders* (ISSN 0960-8966). For 2010 Volume 20 is scheduled for publication. Subscription prices are available upon request from the Publisher. Subscriptions are accepted on a prepaid basis only and are entered on a calendar year basis. Issues are sent by surface mail except to the following countries where air delivery by SAL mail is ensured: Argentina, Australia, Brazil, Canada, Hong Kong, India, Israel, Japan, Malaysia, Mexico, New Zealand, Pakistan, PR China, Singapore, South Africa, South Korea, Taiwan, Thailand, USA. For all other countries airmail rates are available upon request. Claims for missing issues should be make within six months of our publication (mailing) date.

Orders, claims, and journal enquiries: please contact the Elsevier Customer Service Department nearest you:

St. Louis: Elsevier Customer Service Department, 3251 Riverport Lane, Maryland Heights, MO 63043, USA; phone: (800) 6542452 [toll free within the USA]; (+1) (314) 4478871 [outside the USA]; fax: (+1) (314) 4478029; e-mail: JournalsCustomerService-usa@elsevier.com

Oxford: Elsevier Customer Service Department, The Boulevard, Langford Lane, Kidlington, Oxford OX5 1GB, UK; phone: (+44) (1865) 843434; fax: (+44) (1865) 843970; e-mail: JournalsCustomerServiceEMEA@elsevier.com

Tokyo: Elsevier Customer Service Department, 4F Higashi-Azabu, 1-Chome Bldg, 1-9-15 Higashi-Azabu, Minato-ku, Tokyo 106-0044, Japan; phone: (+81) (3) 5561 5037; fax: (+81) (3) 5561 5047; e-mail: JournalsCustomerServiceJapan@elsevier.com

Singapore: Elsevier Customer Service Department, 3 Killiney Road, #08-01 Winsland House I, Singapore 239519; phone: (+65) 63490222; fax: (+65) 67331510; e-mail: JournalsCustomerServiceAPAC@elsevier.com

The Instructions to Authors can be found on the journal's website www.elsevier.com/locate/nmd.

USA mailing notice: Neuromuscular Disorders (ISSN 0960-8966) is published monthly by Elsevier B.V., Radarweg 29, 1043 NX Amsterdam, the Netherlands. Periodical postage paid at Rahway NJ and additional mailing offices.
USA POSTMASTER: Send change of address to *Neuromuscular Disorders*, Elsevier Customer Service Department, 3251 Riverport Lane, Maryland Heights, MO 63043, USA.
AIRFREIGHT AND MAILING in USA by Mercury International Limited, 365 Blair Road, Avenel, NJ 07001.

Funding body agreements and policies: Elsevier has established agreements and developed policies to allow authors whose articles appear in journals published by Elsevier, to comply with potential manuscript archiving requirements as specified as conditions of their grant awards. To learn more about existing agreements and policies please visit http://www.elsevier.com/fundingbodies

Neuromuscular Disorders **has no page charges**

Neuromuscular Disorders 20 (2010) 102–110

Contents lists available at ScienceDirect

Neuromuscular Disorders

ELSEVIER

journal homepage: www.elsevier.com/locate/nmd

Comparative analysis of antisense oligonucleotide sequences targeting exon 53 of the human *DMD* gene: Implications for future clinical trials

Linda J. Popplewell [a,1], Carl Adkin [b,1], Virginia Arechavala-Gomeza [b], Annemieke Aartsma-Rus [c], Christa L. de Winter [c], Steve D. Wilton [d], Jennifer E. Morgan [b], Francesco Muntoni [b], Ian R. Graham [a], George Dickson [a,*]

[a] *School of Biological Sciences, Royal Holloway – University of London, Egham, Surrey TW20 0EX, United Kingdom*
[b] *Dubowitz Neuromuscular Centre, UCL Institute of Child Health, 30 Guilford Street, London WC1N 1EH, United Kingdom*
[c] *Center for Human and Clinical Genetics, Leiden University Medical Center, PO Box 9600, 2300RC Leiden, The Netherlands*
[d] *Centre for Neurological and Neuromuscular Disorders, Australian Neuromuscular Research Institute, University of Western Australia, Perth, WA 6009, Australia*

ARTICLE INFO

Article history:
Received 13 July 2009
Received in revised form 20 October 2009
Accepted 27 October 2009

Keywords:
Exon skipping
Duchenne muscular dystrophy
Antisense oligonucleotides
Phosphorodiamidate morpholino oligomer

ABSTRACT

Duchenne muscular dystrophy (DMD) is caused by the lack of functional dystrophin protein, most commonly as a result of a range of out-of-frame mutations in the *DMD* gene. Modulation of pre-mRNA splicing with antisense oligonucleotides (AOs) to restore the reading frame has been demonstrated in vitro and in vivo, such that truncated but functional dystrophin is expressed. AO-induced skipping of exon 51 of the *DMD* gene, which could treat 13% of DMD patients, has now progressed to clinical trials. We describe here the methodical, cooperative comparison, in vitro (in DMD cells) and in vivo (in a transgenic mouse expressing human dystrophin), of 24 AOs of the phosphorodiamidate morpholino oligomer (PMO) chemistry designed to target exon 53 of the *DMD* gene, skipping of which could be potentially applicable to 8% of patients. A number of the PMOs tested should be considered worthy of development for clinical trial.

1. Introduction

Duchenne muscular dystrophy (DMD) is a severe muscle-wasting disease, affecting 1:3500 live male births, caused by the lack of functional dystrophin protein in skeletal muscles, as a result of frame-disrupting deletions or duplications or, less commonly, nonsense or missense mutations in the *DMD* gene [1]. Mutations that maintain the reading frame of the gene and allow expression of semi-functional, but internally-deleted dystrophin are generally associated with the less severe Becker muscular dystrophy (BMD) [1,2].

Transforming an out-of-frame DMD mutation into its in-frame BMD counterpart with antisense oligonucleotides (AOs) is the basis of the potentially exciting exon skipping therapy for DMD (reviewed by Muntoni and Wells) [3]. The hybridization of AOs to specific RNA sequence motifs prevents assembly of the spliceosome, so that it is unable to recognise the target exon(s) in the pre-mRNA and include them in the mature gene transcript [4,5]. AOs have been used to induce skipping of specific exons such that the reading frame is restored and truncated dystrophin expressed in vitro in DMD patient cells [6,5,7–9], and in animal models of the disease in vivo [4,10–13].

Initial proof-of-principle clinical trials, using two different AO chemistries (phosphorothioate-linked 2′-*O*-methyl modified bases (2′OMePS) [14] and phosphorodiamidate morpholino oligomer (PMO) [15]) for the targeted skipping of exon 51 of the *DMD* gene after intramuscular injection, have been performed recently with encouraging results. While both chemistries have excellent safety profiles [16,17], PMOs appear to produce more consistent and sustained exon skipping in the *mdx* mouse model of DMD [18–20], in human muscle explants [21], and dystrophic canine muscle cells in vitro [22]. However, for some human exons, 2′OMePS and PMO AONs performed equally well [17]. Since the mutations that cause DMD are so diverse, of those DMD patients with genomic deletions, skipping of exon 51 would have the potential to treat only 13% of such patients on the Leiden DMD database [23], and 15% of such patients on the UMD-DMD France mutations database (see http://www.umd.be/DMD/4ACTION/W_MONO). Although any predictions on the frequency of mutations and percentage of skippable patients should be viewed with caution, it is undeniable that the continued development and analysis of AOs for the targeting of other DMD exons is vital.

Here we report the comparative analysis of a series of PMOs targeted to exon 53, skipping of which would have the potential to

* Corresponding author. Tel.: +44 (0)1784 443870; fax: +44 (0)1784 414224. *E-mail address:* g.dickson@rhul.ac.uk (G. Dickson).
[1] These authors contributed equally to this paper.

0960-8966/$ - see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.nmd.2009.10.013

treat a further 8% of DMD patients with genomic deletions on the Leiden database [23], and a further 13.5% of patients on the UMD-DMD France mutations database (see http://www.umd.be/DMD/4ACTION/W_MONO). PMOs designed and previously tested in normal human skeletal muscle cells (hSkMCs) for the targeting of exon 53 [24] were further studied here in cells from a DMD patient with a relevant deletion (del 45–52). These PMOs were directly compared to a PMO based on a AO previously identified as being the most bioactive by Wilton et al. [25]. Time-course studies were performed to evaluate the persistence of skipping and dose-responses were examined. Findings from these experiments were supported by in vivo studies in a mouse model transgenic for the entire human dystrophin locus [12]. Collectively, this work reports a number of PMOs able to produce targeted skipping of exon 53 to levels that would suggest them worthy of consideration for upcoming PMO clinical trials.

2. Materials and methods

2.1. AO design

All AOs were synthesized as phosphorodiamidate morpholino oligomers (PMOs) by Gene Tools LLC (Philomath OR, USA).

2.2. DMD patient primary myoblast culture

Skeletal muscle biopsy samples were taken from a diagnostic biopsy of the quadriceps from a DMD patient with a deletion of exons 45–52. Informed consent was obtained before any processing of samples, and all work was carried out with the approval of the institutional ethics committee. Muscle precursor cells were prepared from the biopsy sample by sharp dissection into 1 mm^3 pieces and disaggregated in solution containing HEPES (7.2 mg/ml), NaCl (7.6 mg/ml), KCl (0.224 mg/ml), glucose (2 mg/ml), Phenol red (1.1 μg/ml), 0.05% Trypsin–0.02% EDTA (Invitrogen, Paisley, UK) in distilled water, three times at 37 °C for 15 min in Wheaton flasks with vigorous stirring. Isolated cells were plated in non-coated plastic flasks and cultured in Skeletal Muscle Growth Media (Promocell, Heidelberg, Germany) supplemented with 10% Foetal Bovine Serum (PAA Laboratories, Yeovil, UK), 4 mM L-glutamine and 5 μg/ml gentamycin (Sigma–Aldrich, Poole, UK) at 37 °C in 5% CO_2.

2.3. Nucleofection of DMD primary myoblasts

Between 2×10^5 and 1×10^6 cells/ml were pelleted and resuspended in 100 μl of solution V (Amaxa Biosystems, Cologne, Germany). The appropriate PMO to skip exon 53 was added to the cuvette provided, sufficient to give the concentrations described, followed by the cell suspension, and nucleofected using the Amaxa nucleofector 2, program B32. Five hundred microliters of medium was added to the cuvette immediately following nucleofection [26]. This suspension was transferred to a 6 well plate in differentiation medium. Nucleofected cells were maintained in differentiation media for 3–21 days post treatment before extraction of RNA or protein. Transfections were performed blindly and in each experiment in triplicate. Each experiment was repeated at least once to ensure reproducibility of results.

2.4. Lactate dehydrogenase cytotoxicity assay

A sample of medium was taken 24 h post-transfection to assess cytotoxicity by release of lactate dehydrogenase (LDH) into the medium, using the LDH Cytotoxicity Detection Kit (Roche, Burgess Hill, UK), following the manufacturer's instructions. The mean of three readings for each sample was recorded, with medium only, untreated and dead controls. The readings were normalised for background (minus medium only) and percentage toxicity expressed as [(sample-untreated)/(dead-untreated) × 100].

2.5. Transgenic human DMD mice

A transgenic mouse expressing a complete copy of the human *DMD* gene has been generated [12,27]. Experiments were performed at the Leiden University Medical Center, with the authorization of the Animal Experimental Commission (UDEC) of the Medical Faculty of Leiden University as described previously [9]. Twenty micrograms of each PMO was injected once into two gastrocnemius muscles, pretreated with cardiotoxin. Mice were sacrificed 1 week after the injection, and RNA harvested from the isolated gastrocnemius muscles and analysed by RT-PCR.

2.6. RNA isolation and reverse transcription-polymerase chain reaction analysis

RNA was isolated and analysed by RT-PCR, as described previously [9]. Primer sequences and detailed PCR protocols are available on request. PCR products were analysed on 1.5% (w/v) agarose gels in Tris–borate/EDTA buffer. Skipping efficiencies were determined from gel images by comparing induced shortened dystrophin mRNAs to the intact transcript of the full length using densitometric analysis with Image J software (for patient samples) or by quantifying the skipped products with DNA 1000 LabChip Kit on the Agilent 2100 bioanalyzer (Agilent Technologies, USA) (for hDMD mouse samples). Skipping percentages were calculated as the amount of skip transcripts relative to the total transcripts (skip and full length). Equal amounts of the induced and intact transcripts would be regarded as representing 50% efficiency, while an estimate of 25% exon skipping would be represented by the intact transcript being three times more abundant than the band representing the induced transcript. Likewise, if the induced transcript was present at three times the level of the intact transcript, the exon skipping efficiency would be assessed to be 75%. Where appropriate, the two-tailed student's *t*-test was used to determine the statistical strength of the skipping efficiencies produced.

2.7. Sequence analysis

RT-PCR products were excised from agarose gels and extracted with a QIAquick gel extraction kit (Qiagen, Crawley, UK). Direct DNA sequencing was carried out by the MRC Genomics Core Facility.

2.8. Western blot analysis of dystrophin protein

DMD patient cells, transfected as described and cultured in differentiation medium, were harvested 7, 14 or 21 days post-transfection. Cells (4×10^5) were pelleted and resuspended in 50 μl of loading buffer (75 mM Tris–HCl pH 6.8, 15% sodium dodecyl sulphate, 5% ß-mercaptoethanol, 2% glycerol, 0.5% bromophenol blue and complete mini protease inhibitor tablet). Samples were incubated at 95 °C for 5 min and centrifuged at 18,000g for 5 min. Twenty microliters of sample was loaded per well in a 6% polyacrylamide gel with 4% stacking gel. Protein from CHQ5B cells differentiated for 7 days was used as a positive control for dystrophin. Gels were electrophoresed for 5 h at 100 V before blotting on nitrocellulose membrane at 200 mA overnight on ice. Blots were stained with protogold to assess protein loading, then blocked in 10% non-fat milk in PBS with 2% Tween (PBST) for 3 h. Blots were probed with antibodies to dystrophin, NCL-DYS1 (Vector Labs, Peterborough, UK) diluted 1:40 and to dysferlin, Hamlet1 (Vector Labs)

diluted 1:300 in 3% non-fat milk/PBST. An anti-mouse, biotinylated secondary antibody (diluted 1:2000; GE Healthcare, Amersham, UK) and streptavidin/horse radish peroxidise conjugated antibody (1:10,000; Dako, Ely, UK) allowed visualisation in a luminol-HRP chemiluminescence reaction (ECL-Plus; GE Healthcare) on Hyperfilm (GE Healthcare), exposed at intervals from 10 s to 4 min.

2.9. Statistical analysis

For the blind comparison at 300 nM in DMD patient cells, data from two separate experiments performed in duplicate and triplicate respectively were pooled and compared by two-tailed student *t*-test. Dose–response and time-course experiments were compared by two-tailed, paired *t*-test.

3. Results

Twenty-four AOs designed to target exon 53 of the *DMD* gene have been previously tested in normal human skeletal muscle cells (hSkMCs) [24,25]. Table 1 summarises the names and target sequence characteristics of these AOs (shown in bold), and % skipping produced by each in normal hSkMCs. However, studies in normal hSkMCs are limited as they do not allow assessment of the therapeutic effect at the protein level (i.e. dystrophin restoration). Further studies have therefore been performed here to elucidate and confirm which AO(s) would have the potential as a treatment for patients with an eligible deletion. AOs, whose target sites are within the sequence +29 to +74 of exon 53, the region previously shown to be in open conformation, binding to which interferes with spliceosome-mediated pre-mRNA splicing, such that exon 53 is skipped [24,25], were directly compared in exon 53-skippable patient cells (at UCL), and in the humanised DMD (hDMD) mouse (at LUMC). The AOs were all synthesized as PMOs to allow direct comparison of skipping efficacy. While PMOs were hybridized to mixed-backbone DNA leashes in the previous study [24], the nucleofection method used here was performed on unleashed PMOs.

3.1. Comparison of PMOs to exon 53 in DMD patient cells

Our comparative evaluation of PMO-induced exon skipping efficiencies was performed in a blinded fashion. All transfections were performed in triplicate and repeated at least once to ensure uniformity of results. Skipping efficiencies were determined from RT-PCR gel images by comparing induced shortened dystrophin mRNAs to the intact full length transcript using densitometric analysis, as described previously [25]. Sequencing of RT-PCR products confirmed the targeted skipping of exon 53 (results not shown). For quantification, the skip-products were analysed using densitometric analysis with Image J software. This technique for quantifying skipping efficiencies of AOs targeted to the *DMD* gene has been published previously [9,17]. Real-time PCR quantification of intact and induced transcripts has proven to be impossible due to a number of obstacles (variation of amplification efficiencies of each transcript, possible interference of intact and induced transcript primers/probes with each other) (results not shown). No DMD exon skipping studies thus far reported have included real-time PCR quantification of AO efficacy, and we believe we have used the best method available for quantification. Skipping efficiency is given as the percentage of skip transcript over the total amount of transcript (skip and full length). AOs were sub-divided on the basis of their skipping efficiency. PMOs that produced over 50% exon skipping were designated as Type 1, those that produced between 25% and 50% exon skipping were described as Type 2, while those that produced less than 25% as Type 3. Where appropriate, the two-tailed student's *t*-test was used to assess significant differences between AOs.

The 13 PMOs, whose target sites are within the sequence +29 to +74 of exon 53, were compared directly at a 300 nM dose by nucleofection [26]. This dose was selected for comparison, since such concentrations of AOs have been used in numerous previous exon skipping studies in DMD [5,6,9]. PMOs-G, -H and -A were the most efficient, producing a mean of 73% (±4.10%), 68% (±4.77%) and 68% (±4.14%) exon skipping respectively (classified as Type 1) (Fig. 1). The other PMOs tested produced the following exon skipping levels: PMO-I, 63% (±7.5%); PMO-B, 56% (±6.29%); PMO-M, 52% (±10.78%) (all classified as Type 1); PMO-J, 37% (±4.95%); (classified as Type 2). All other PMOs tested gave exon skipping at levels of between 15% and 26%. When compared by two-tailed student *t*-test, PMO-G (the most efficient PMO) gave significantly higher levels of exon skipping than PMOs -C ($p < 0.0001$), -D ($p < 0.0001$), -E ($p < 0.0001$), -F ($p < 0.0001$), -J ($p = 0.0005$), -K ($p = 0.0002$) and -L ($p < 0.0001$), but was not significantly more effective than the other PMOs tested. The more efficacious PMOs should produce sustained and pronounced exon skipping when applied at lower concentrations. Therefore, the six most effective PMOs (i.e. Type 1) (-A, -B, -G, -H, -I and -M) were selected for dose–response and time-course experiments.

When the concentration dependence of exon skipping was examined for the most efficient PMOs, skipping levels approaching 30% were evident for the Type 1 PMOs -G and -H at concentrations as low as 25 nM (Fig. 2a, b). The other PMOs classified as Type 1 (PMOs -A, -B, -I and -M) did not induce such levels of exon skipping when used at lower concentrations. Similar levels of skipping (30%) were only achieved by PMO-A, PMO-B and PMO-M at 100 nM, while PMO-I needed to be present at 200 nM to produce over 30% exon skipping (Fig. 2a, b). This is why the concentration dependence of exon skipping is a valuable tool in ascertaining the most efficient AO(s).

The exon skipping produced by the six Type 1 PMOs was shown to be persistent, lasting for up to 10 days after transfection, with over 60% exon skipping observed for the lifetime of the cultures for PMOs -A, PMO-G and PMO-H (Fig. 3a, b). When compared by two-tailed, paired *t*-test across all time-points, PMO-G gave significantly higher levels of skipping than PMOs -B, -H, -I and -M ($p = 0.0004$, 0.0126, 0.0008 and 0.0004, respectively) and bordered on significance for PMO-A ($p = 0.0550$). Three of the most effective Type 1 PMOs (-A, -G and -H) were also compared in a longer time-course experiment up to 21 days after transfection (Fig. 3c, d). PMO-G gave sustained high levels of exon skipping (over 60%) for the 21 days whereas skipping by PMOs -A and -H had fallen to 48% and 46%, respectively by day 17. When compared by two-tailed, paired *t*-test across all time-points, PMO-G gave significantly higher levels of skipping than PMOs -A and -H ($p = 0.0422$ and 0.0231, respectively). There was no evidence that any of the PMOs tested caused cellular cytotoxicity relative to mock-transfected controls, as assessed by visual inspection, and lactate dehydrogenase release into culture medium (results not shown). The relative efficacy of the six Type 1 PMOs in the direct comparison, dose–response and time-course assays is summarised in Fig. 4a. Exon 53 skipping by PMO-G is used as the baseline set at 100%. This clearly shows the PMO-G outperforms the other Type 1 PMOs in vitro in patient cells. However, it should also be noted that a number of the other Type 1 PMOs, namely PMO-A and PMO-H, also appear to perform very well across these three comparative tests.

The most effective Type 1 PMO (PMO-G) from the time-course experiments was compared to the most effective PMO to skip exon 51 that has been taken forward to clinical trial, in DMD patient cells with an amenable mutation (del 45–52 for exon 53 skipping and del 48–50 for exon 51 skipping) in a dose–response experiment from 25 to 400 nM (see Fig. 4b). PMO-G gave higher levels

Table 1
Table summarising the characteristics of PMOs used. Characteristics of the PMOs and their target sites listed (taken from [24]).

	PMO	% Skip	Position Start	Position End	%GC	Exon-PMO binding energy	PMO-PMO binding energy	% Open[a]	Ends in open loops[a]	% Overlap with hybrid. peak
(a)										
A	h53A1	12.7	+35	+59	52	−38.6	−17.4	50	2	92
B	h53A2	9.7	+38	+62	56	−36.1	−17.4	46.7	1	100
C	h53A3	2.0	+41	+65	56	−36.7	−13.7	36.7	0	0
D	h53A4	10.5	+44	+68	48	−34.3	−8.5	20	0	100
E	h53A5	9.0	+47	+71	48	−35.5	−8.5	43.3	2	100
F	h53A6	0.3	+50	+74	48	−35.3	−8.5	43.3	2	92
N	h53B1	0	+69	+93	28	−22.1	−12.1	53.3	1	0
O	h53B2	0.6	+80	+104	48	−30.1	−11.3	23.3	1	0
P	h53B3	3.0	+90	+114	48	−34.5	−5.5	48	2	0
Q	h53C1	0	+109	+133	48	−32.4	−9.8	46.7	2	0
R	h53C2	0	+116	+140	56	−31.3	−12.7	33.3	1	0
S	h53C3	0	+128	+152	60	−34.6	−13.7	26.7	1	0
T	h53D1	0	+149	+173	52	−34.1	−13.4	30	1	0
U	h53D2	0.9	+158	+182	48	−36.5	−14.5	40	2	0
V	h53D3	3.7	+170	+194	36	−34.3	−11.2	40	1	0
W	h53D4	12.3	+182	+206	32	−30.9	−9.2	63.3	1	0
X	h53D5	7.9	+188	+212	36	−31.5	−3.3	66.7	1	0
G	h53A30/1	52.4	+30	+59	50	−48.1	−17.4	56.7	1	92
H	h53A30/2	87.2	+33	+62	53	−45.1	−17.4	63.3	1	100
I	h53A30/3	80.1	+36	+65	53	−44.6	−17.4	53.3	1	100
J	h53A30/4	38.6	+39	+68	50	−43.4	−17.4	43.3	1	100
K	h53A30/5	9.4	+42	+71	47	−42.4	−11.3	46.7	1	100
L	h53A30/6	35.9	+45	+74	47	−42.3	8.5	56.7	1	100
M	H53A	N/D	+39	+69	52	−48.5	−17.4	48.4	2	100

	PMO	# Rescue ESE sites	% Overlap with Rescue ESE	% Overlap with PESE	% Overlap with PESS	ESE finder values over threshold[b] SF2/ASF	BRCA1	SC35	SRp40	SRp55	Tra2B	9G8
(b)												
A	h53A1	7	56	84	0	6.58	7.26	0	3.12	0	24.04	19.02
B	h53A2	4	32	72	0	6.58	7.26	0	3.12	0	7.25	19.02
C	h53A3	3	32	60	0	6.58	7.26	0	3.12	0	7.25	11.9
D	h53A4	4	28	48	8	6.58	7.26	0	3.12	0	7.25	11.9
E	h53A5	3	36	36	20	6.58	7.26	0	3.12	0	7.25	11.9
F	h53A6	2	36	28	32	6.58	7.26	0	0	0	7.25	11.9
N	h53B1	5	56	40	40	0	9.26	3.62	10.66	0	5.06	1.1
O	h53B2	5	60	60	0	0	9.26	3.62	4.73	0	5.06	8.28
P	h53B3	8	72	64	0	3.49	9.26	3.44	4.73	0	24.04	28.68
Q	h53C1	6	52	72	0	4.19	6.72	0	2.04	0	24.04	28.68
R	h53C2	1	24	60	0	4.19	6.72	10.2	4.38	0	0	8.28
S	h53C3	1	24	32	0	3.49	6.41	10.2	4.38	6.86	0	14.18
T	h53D1	4	40	32	0	0.52	0	18.68	0	6.86	0	12.71
U	h53D2	6	44	32	0	0.52	1.8	18.68	0.42	0	0	12.71
V	h53D3	9	64	0	0	0	1.8	0	6.95	0	24.04	10.49
W	h53D4	16	96	24	0	8.5	11.95	0	7.67	0.33	24.04	7.14
X	h53D5	14	92	44	0	8.5	11.95	0	7.67	0.33	24.04	7.14
G	h53A30/1	9	60	86	0	6.58	7.26	0	3.12	0	24.04	19.02
H	h53A30/2	8	53	77	0	6.58	7.26	0	3.12	0	24.04	19.02
I	h53A30/3	6	43	67	0	6.58	7.26	0	3.12	0	24.04	19.02
J	h53A30/4	4	43	57	7	6.58	7.26	0	3.12	0	7.25	11.9
K	h53A30/5	5	47	47	17	6.58	7.26	0	3.12	0	7.25	11.9
L	h53A30/6	5	48	37	27	6.58	7.26	0	3.12	0	7.25	11.9
M	H53A	4	45	58	10	6.58	7.26	0	3.12	0	7.25	11.9

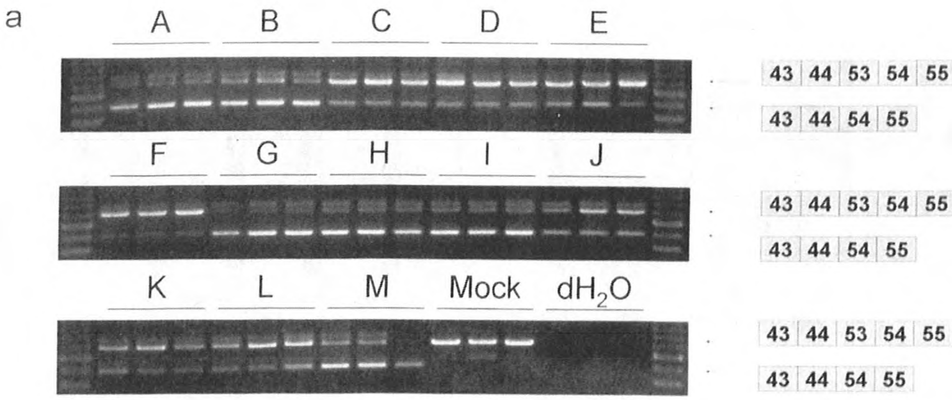
Alphabetical codes shown in bold represent the PMOs compared in this present study.
[a] Calculated as % of PMO target site in open structures on predicted RNA secondary structure obtained using MFOLD analysis. The position of the PMO target sites relative to open loops in the RNA secondary structure is listed (0, no ends in open loops; 1, one end in an open loop; 2, both ends in open loops).
[b] In our analyses, SR binding sites were predicted using splice sequence finder (http://www.umd.be/SSF/) software. Values above threshold are given for PMOs whose target sites cover 50% or more of potential binding sites for SF2/ASF, BRCA1, SC35, SRp40, SRp55, Tra2β and 9G8.

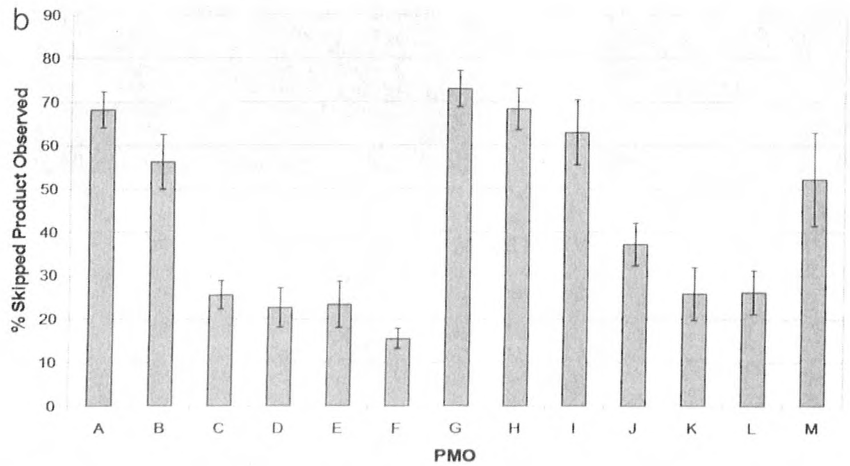
of skipping at all doses tested. When compared by two-tailed, paired *t*-test, PMO-G across all concentrations used, gave significantly higher levels of skipping than h51A ($p = 0.0033$).

Western blot analysis of DMD patient (del 45–52) cell lysates, treated in culture with the Type 1 25mers (PMOs -A and-B) and 30mers (-G, -H, -I and -M) is shown in Fig. 5a. De novo expression of dystrophin protein was evident with all six PMOs, but was most pronounced with PMOs -H, -I, -G and -A, producing 50%, 45%, 33% and 26% dystrophin expression, respectively, relative to the positive control, and seemingly weakest with PMO-B and PMO-M (11% and 17% dystrophin expression respectively, relative to the positive control). Although there are limitations to quantifying Western blots of this nature, the qualitative importance of the data holds.

3.2. Comparison of PMOs to exon 53 in humanised DMD mouse

The hDMD mouse is a valuable tool for studying the processing of the human *DMD* gene in vivo, and as such provides a model for studying the in vivo action of PMOs, prior to clinical testing in

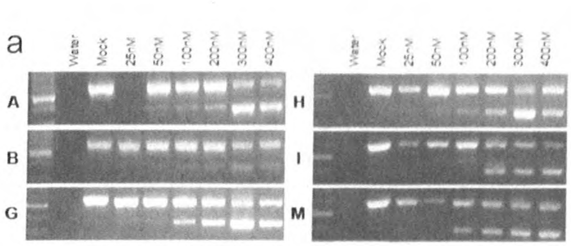


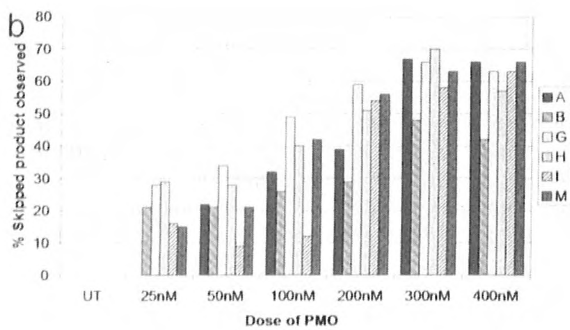


c

PMO	P-value
G vs A	0.4496
G vs B	0.0577
G vs C	<0.0001*
G vs D	<0.0001*
G vs E	<0.0001*
G vs F	<0.0001*
G vs H	0.4935
G vs I	0.2799
G vs J	0.0005*
G vs K	0.0002*
G vs L	<0.0001*
G vs M	0.1124

Fig. 1. Blind comparison of 13 PMO oligonucleotide sequences to skip human exon 53. Myoblasts derived from a DMD patient carrying a deletion of dystrophin exons 45–52 were transfected by nucleofection with each of the PMOs (300 nM) in triplicate. RNA was harvested 3 days following transfection, and amplified by nested RT-PCR. (a) Bars indicate the percentage of exon skipping achieved for each PMO, derived from Image J analysis of the electropherogram of the agarose gel (b). Skipped (477 bp) and unskipped (689 bp) products are shown schematically. The larger full length amplicon is often seen and is due to carry over of primers from the first round of the PCR into the second. (c) Efficacy of PMOs was compared by two-tailed, student *t*-test. PMO-G gave significantly higher efficacy of exon skipping than PMOs C, D, E, F, J, K and L ($^*p < 0.05$), but not significantly higher than PMOs A, B, H, I and M.

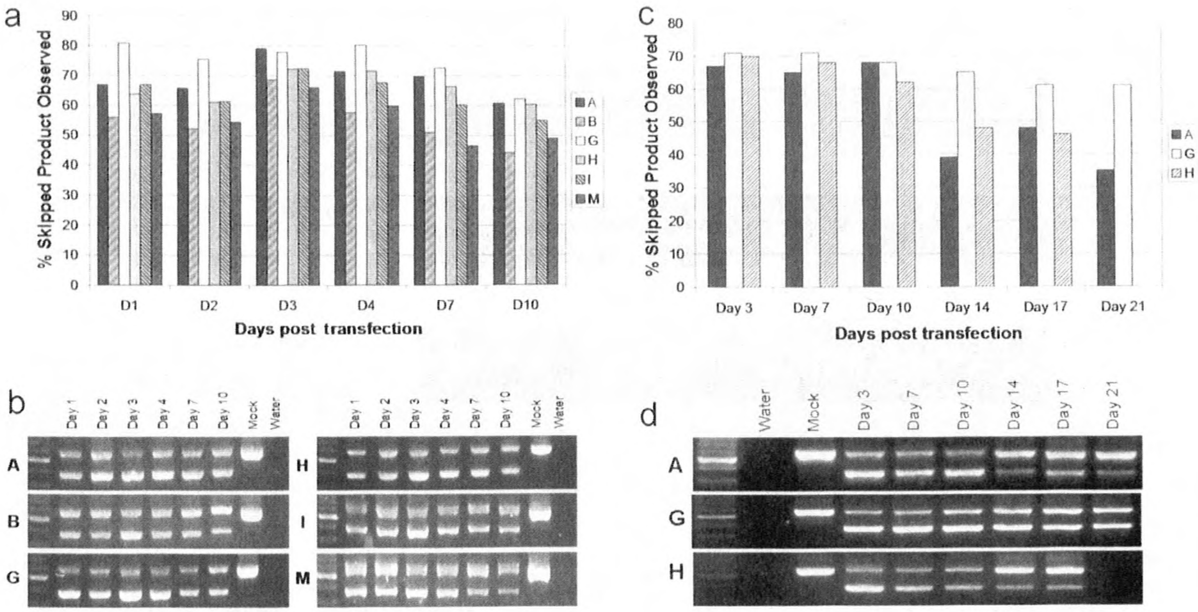




c

PMO	P-value
G vs A	0.0491*
G vs B	0.0022*
G vs H	0.0941
G vs I	0.0527
G vs M	0.0759

Fig. 2. Dose–response of the six best-performing PMOs. (a) Myoblasts derived from a DMD patient carrying a deletion of dystrophin exons 45–52 were transfected with the six best-performing PMOs by nucleofection, at doses ranging from 25 to 400 nM. RT-PCR products derived from RNA isolated from cells 3 days post-transfection were separated by agarose gel electrophoresis. (b) The percentage of exon skipping observed is expressed for each concentration of each PMO as a comparison of the percentage OD of skipped and unskipped band, as measured using Image J. (c) Data for each PMO over the range of doses were pooled and compared by two-tailed, paired student *t*-test. PMO-G gave significantly higher efficiency than PMOs A and B ($^*p < 0.05$) but did not give significantly higher efficacy than PMOs H, I and M.



e

PMO	P-value (10 days)	P-value (21 days)
G vs A	0.0550	0.0422*
G vs B	0.0004*	-
G vs H	0.0126*	0.0231*
G vs I	0.0008*	-
G vs M	0.0004*	-

Fig. 3. Persistence of dystrophin expression in DMD cells following PMO treatment. (a) Myoblasts derived from a DMD patient carrying a deletion of dystrophin exons 45–52 were transfected by nucleofection with each of the six best-performing PMOs (300 nM), and were cultured for 1–10 days before extracting RNA. The percentage of exon skipping was compared using the percentage OD of skipped and unskipped bands, measured using Image J analysis of the agarose gel of the nested RT-PCR products shown in (b). (c) Long term exon skipping up to 21 days after transfection with PMOs A, G and H. The percentage of exon skipping was compared using the percentage OD of skipped and unskipped bands, measured using Image J analysis of the agarose gel of the nested RT-PCR products shown in (d). (e) Data for each PMO over the time-course experiments were pooled and compared by two-tailed, paired student *t*-test. Over the 10 day time-course experiment, PMO-G gave significantly higher efficiency than PMOs B, H, I and M ($^*p < 0.05$) but did not give significantly higher efficacy than PMO A. Over the 21 day time-course experiment, PMO-G gave significantly higher efficacy than PMOs A and H ($^*p < 0.05$).

patients. The Type 1 PMOs (-A, -G, -H, -I and -M) (20 μg) were injected into the gastrocnemius muscle of hDMD mice in duplo. RNA extracted from the muscles was analysed for exon 53 skipping by RT-PCR (Fig. 5b). For quantification, the skipped products were analysed using the DNA 1000 LabChip Kit on the Agilent 2100 bioanalyzer, which, unlike densitometry, corrects for fragment length. Skipping percentages were calculated as the amount of skip transcripts relative to the total transcripts (skipped and full length). Skipping of exon 53 was evident for each of the PMOs tested; average skipping seen in both legs was 8% for PMO-A, 7.6% for PMO-I, 7.2% for PMO-G, but a slightly lower level of 4.8% for PMO-H. PMO-M produced exon skipping levels of less than 1%, which is the detection threshold for the system used.

4. Discussion

We describe here the comparative analysis of PMOs designed to target exon 53 of the human *DMD* gene and thereby induce its skipping. Previously, a series of PMOs had been designed and their exon skipping efficacy investigated in normal human skeletal muscle cells [24]. These were directly compared to a PMO based on an AO previously identified as being the most bioactive by Wilton et al. [25]. Skipping efficiencies of the PMOs were compared here by two independent groups in two different systems (at UCL and LUMC). Such a collaborative approach has been used previously as a way of validating target sequences in DMD [9]. The use of primary human myoblast cultures allowed controlled in vitro comparison of PMO sequences, and confirmation of skipping of exon 53 at the RNA level by PMOs in DMD patient cells with a relevant mutation. These results were further borne out by the expression of dystrophin protein in the DMD cells treated with specific PMOs. Use of the humanised DMD mouse provided an in vivo setting to confirm correct exon exclusion prior to any planned clinical trial. The combined use of these three different systems (normal cells, patient cells and hDMD mouse) as tests of PMO bioactivity provided a reliable and coherent confirmation of optimal sequence(s) for the targeted skipping of exon 53.

The putative use of AOs to skip the exons which flank out-of-frame deletions is fast becoming a reality in the treatment of DMD boys. Indeed the restoration of dystrophin expression in the TA muscle of four patients, injected with a 2′OMePS AO optimised to target exon 51 of the *DMD* gene, has been reported recently [14]. Moreover a clinical trial using a PMO targeting exon 51 has recently been completed in seven DMD boys in the UK [15]. However, the targeted skipping of exon 51 would have the potential to treat only 13% of DMD patients on the Leiden database with genomic deletions [23]. There is therefore a definite requirement for the optimisation of AOs to target other exons commonly mutated in DMD. The targeted skipping of exon 53 of the human

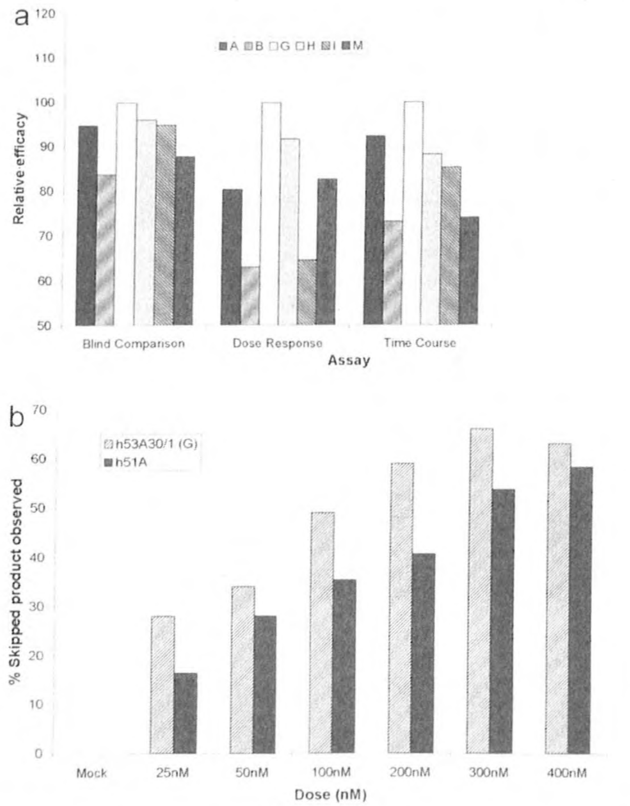


Fig. 4. Relative efficacy of Type 1 PMOs for the targeted skipping of exon 53. (a) The relative efficacy of the 6 most effective PMOs compared in the different assays. PMO-G was used as a baseline set at 100%. (b) The most effective PMO (G) compared to the most effective PMO to skip exon 51 in amenable DMD patients cells at doses ranging from 25 to 400 nM. PMO-G gave significantly higher levels of skipping (p = 0.0033 compared by two-tailed, paired t-test).

DMD gene would have the potential to treat a further 8% of DMD patients on the Leiden database [23], and 13.5% of patients on the UMD-DMD France mutations database (see http://www.umd.be/DMD/4ACTION/W_MONO).

There have been many large screens of AO bioactivity in vitro [24,25,28,29] which provide guidelines to aid AO design. The targeting of AOs to exonic splicing enhancer (ESE) motifs [25,28,29], RNA secondary structure, target site accessibility and strength of AO-target binding are all important predictors of AO efficacy [24,29]. Although there are tools available to aid the design of AOs for the targeted skipping of *DMD* exons, the empirical analysis of AOs is still required. Hence the importance of this study in the development of AO sequences as potential gene therapy drugs for DMD. The data presented here would indicate that PMOs targeting within the sequence +30+65 of exon 53 (namely PMO-A, -G and -H) produce levels of exon skipping that may be considered effective (over 50% exon skipping). There remains however the possibility that a stepped base-by-base screening of AOs across the entirety of exon 53 and some indeterminate distance into the flanking intronic sequences might reveal an AO with a better dose–response and longevity of action profile. Sequence +30+65 has been shown to be accessible to binding on hexamer hybridization array analysis and in open conformation by mfold prediction of pre-mRNA secondary structure [24]. These Type 1 PMOs can therefore bind more strongly since they can access their target site more directly. These thermodynamic considerations have also been reported in a complementary study of 2′OMePS AOs [29]. Indeed, the fact that the 30mer PMOs (-G, -H) were more bioactive than 25mer PMO counterpart (-A) targeted to the same open/accessible sites on the exon, would suggest that strength of binding of PMO to the target site may be the most important factor in determining PMO bioactivity. The influence of AO length on bioactivity has been reported elsewhere [9,30], and is further confirmed in the present study; all 30mers tested were more bioactive relative to their 25mer counterpart. Previous studies by the Leiden

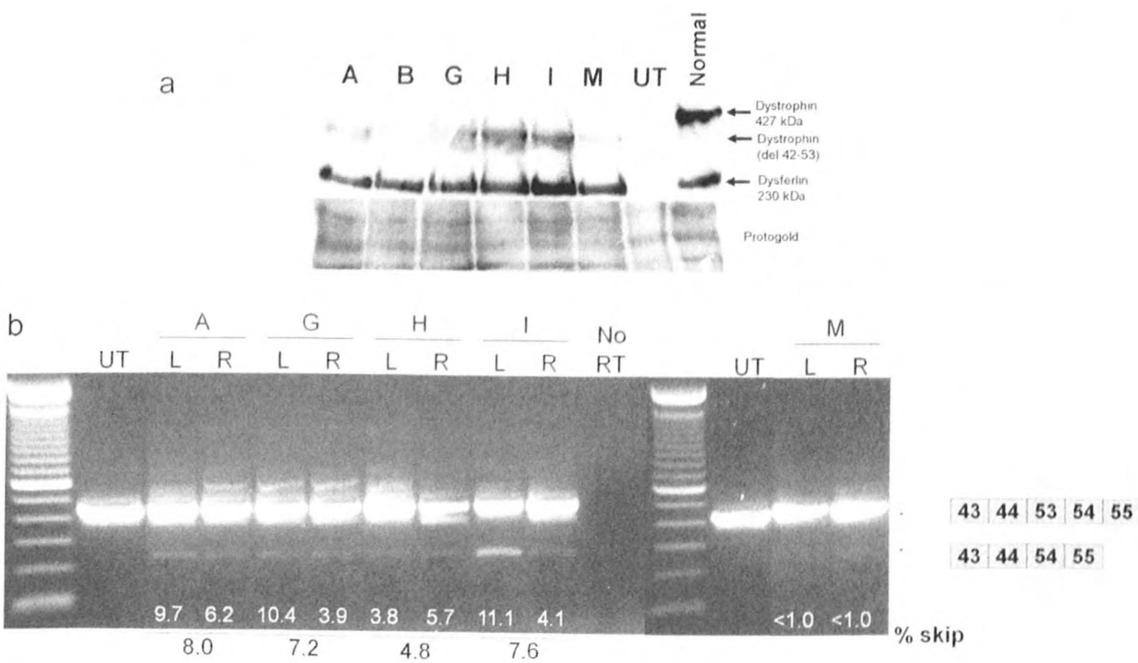


Fig. 5. Analysis of induced dystrophin protein expression in del DMD cells and in vivo efficacy of Type 1 PMOs in hDMD mice. (a) Western blot analysis was performed on total protein extracts from del 45–52 DMD cells 7 days after transfection with the six best PMOs (300 nM). Blots were probed with antibodies to dystrophin, to dysferlin as a muscle-specific loading control, and protogold for total protein loading control. CHQ5B myoblasts, after 7 days of differentiation were used as a positive control for dystrophin protein (normal). (b) PMOs (20 μg) were injected in a blind experiment into the gastrocnemius muscle of hDMD mice in duplo. RT-PCR analysis of RNA harvested from muscle isolated 1 week after injection was performed and products visualised by agarose gel electrophoresis. Quantification of PCR products was performed using a DNA LabChip.

group [28] suggest that the optimal 2′OMePS AO is targeted to the sequence +46+63 of exon 53, producing exon skipping in up to 25% of transcripts in cultured control cells and 7% in the hDMD mouse. This 2′OMePS AO shows some degree of overlap with the optimal PMOs reported here which strengthens our findings. The reason that our PMOs produce higher levels of exon skipping could be a (combined) consequence of the different AO chemistries, length of AO used, type of cell used (patient vs. control) and the absolute target site of AO. No direct comparison was made here between PMO and 2′OMe AOs targeted to the same sequence of exon 53, since the purpose of this study was not to elucidate which chemistry was superior, but to ascertain the optimal target site for a PMO. A direct comparison would in fact be difficult as 2′OMe AOs are generally only 20 nucleotides long, whereas the PMOs used here were 25 and 30mers.

A PMO can be classified as an effective AO if it produces strong (over 50%), consistent and sustained exon skipping after administration to myogenic cells in vitro at low concentrations (25 nM). An AO reagent that produces sustained exon skipping at low concentrations would be preferable in the clinical setting as a gene therapy as this would lower the cost of treatment, extend the therapeutic action of exon skipping and reduce the possible toxicity of chronic AO administration. The levels of skipping produced in vitro by those PMOs targeting the sequence +30+65 are comparable, or indeed superior, to those reported pre-clinically for PRO051 [28] and AVI4658 [9], the 2′OMe and PMO AOs that are now both being used in Phase I/II clinical trials with encouraging results [14,15]. Indeed we directly demonstrate here the greater skipping efficacy of PMO-G relative to AVI4658 (H51A) over a range of concentrations (see Fig. 4b). However predicting the amount of skipping needed in vitro for an AO to be therapeutic in a patient is impossible; the efficiency of exon skipping is likely to differ from patient to patient and mutation to mutation, and the levels of dystrophin protein restoration will depend on the quality of the muscle itself when a clinical treatment is started.

When considering the data presented previously [24] and here as a whole, the superiority of the PMOs targeting the sequence +30+65 (i.e. PMOs -A, -B, -G and -H) is strongly indicated. The 30mers PMO-G and PMO-H produce higher levels of skipping relative to the 25mers PMO-A and PMO-B. In normal myoblasts, liposomal-carrier mediated transfection of leashed forms of these 30mer PMOs targeting produced over 50% skipping of exon 53, implying that they act extremely efficiently within the cell. This was confirmed in patient myoblasts using nucleofection as the entry method of naked PMOs into the cells. The different levels of exon skipping seen here in the patient cells relative to control cells is due to the different concentrations used (300 nM in patient cells versus 500 nM in normal cells), the different techniques used to introduce the PMOs into the cells, and the differences between the cells themselves. The dystrophin in patient cells is generally more readily skippable than normal cells [5,6,31]. The results seen in patient cells were reproducible, implying that sufficient PMO is getting into the cells to induce such skipping, and the order of efficiency of exon skipping induced in patient cells mirrored that seen in normal cells (Table 1, and Fig. 1). Further, these PMOs generate the highest levels of exon skipping in patient cells over a range of concentrations (up to 200 nM) and, most important for potential therapeutic application, exert their activity at concentrations as low as 25 nM. The exon skipping activity of these PMOs is also persistent, with over 60% exon skipping for 21 days in culture for PMO-G. This would have important safety and cost implications as a genetic therapy for DMD patients with the appropriate deletions. PMOs targeting the sequence +30+65 of exon 53 of the *DMD* gene were also shown to skip exon 53 correctly in vivo in the hDMD mouse. It should be noted that the levels of exon skipping in the hDMD mouse by each particular PMO was variable. This has been reported previously [12], and is likely to be due to the poor uptake into the non-dystrophic muscle of the hDMD mouse. However this does not compromise the importance of the finding that the PMOs tested here are able to elicit the targeted skipping of exon 53 in vivo. A summary of the relative efficacy of the different Type 1 PMOs tested over the different assays used is presented in Fig. 4a. The recommendation of PMO-G as a potential clinical trial reagent of choice for the targeted skipping of exon 53 of the *DMD* gene relative to the other Type 1 PMOs, is based primarily on it's more persistent longevity of action. Repeated administration of PMO will be required for prolonged antisense therapeutic action, and the prolonged action of PMO-G makes it an attractive choice with PMOs-A and -H providing viable alternatives if required. These RNA results were further confirmed by the detection of dystrophin protein in extracts from patient cells treated with these PMOs.

Although efficiency of exon skipping is perhaps the most important quality an AO can possess, it is not the only one. The potential for the PMOs targeting sequence +30+65 of exon 53 to have off-target effects could be considered to be negligible, since no completely homologous sequences were found on BLAST analysis (results not shown). There is a common single nucleotide polymorphism (SNP) seen on exon 53 of the *DMD* gene. PMOs -J, -K, -L and -M had this SNP (c7728C>T) in the last, fourth to last, seventh to last and second to last base of their target sites, respectively. There is the potential that this allelic mismatch could influence the binding and bioactivity of these PMOs. However, the more active PMOs (-A, -B, -G, -H and -I) all had their target sites away from the SNP, thus removing the possible effect of a mismatch weakening binding and bioactivity, allowing definitive comparisons between these PMOs to be made. The DMD patient (del 45–52) carried the normal (T) allele, hence the SNP would not affect the binding of the PMOs that anneal at this site. Additionally, there was no evidence that the PMOs produced cellular cytotoxicity (results not shown). This, together with the predicted stability of the PMO–target complexes [24], suggests these PMOs have potential as a clinical therapy.

We would therefore recommend that PMOs targeting sequence +30+65 of exon 53 of the *DMD* gene worthy of consideration for any upcoming clinical trial. In this study, sequence +30+65 was effectively targeted by PMOs-A, -B, -G and -H, resulting in exon 53 skipping. Since repeated delivery would be required for therapeutic action, the more persistent action of PMO-G may suggest this to be the PMO of choice for the targeted skipping of exon 53, and PMOs-A and -H providing viable alternatives if required.

Acknowledgements

The authors wish to thank members of the UK Muscular Dystrophy Exon skipping (MDEX) Consortium for helpful discussions, and the Muscular Dystrophy Campaign, Action Duchenne (formerly Parent Project UK), Duchenne Family Support Group, Muscular Dystrophy Ireland, NIH, Muscular Dystrophy Association USA, Clinigene EC Network of Excellence (LSHB-CT-2006018933) and participating patients for supporting this study. All work was carried out with informed consent and with the approval of the institutional ethics committee. The authors would also like to thank Prof. Ryszard Kole for his advice and careful reading of the paper. The authors also thank Janet Rose Christensen and Peter O'Hanley for the support of AVI Biopharma in the supply of PMO AOs. The work was funded by the Department of Health (UK). JEM was supported by a MRC collaborative career development fellowship in stem cell research and is currently funded by a Wellcome Trust University Award. CA was funded by the Muscular Dystrophy Campaign, Association Francais Contre les Myopathies, EU MYOAMP and is currently funded by the Duchenne Parent Project. The MRC Neuro-

muscular Centre support to the Biobank is also gratefully acknowledged.

References

[1] Hoffmann EP, Brown RH, Kunkel LM. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell 1987;51:919–28.

[2] Monaco AP, Bertelson CJ, Liechti-Gallati S, Moser H, Kunkel LM. An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. Genomics 1988;2:90–5.

[3] Muntoni F, Wells D. Genetic treatments in muscular dystrophies. Curr Opin Neurol 2007;20:590–4.

[4] Mann CJ, Honeyman K, Cheng AJ, et al. Antisense-induced exon skipping and synthesis of dystrophin in the mdx mouse. Proc Natl Acad Sci USA 2001;98:42–7.

[5] Aartsma-Rus A, Janson AA, Kaman WE, et al. Therapeutic antisense-induced exon skipping in cultured muscle cells from six different DMD patients. Hum Mol Genet 2003;12:907–14.

[6] van Deutekom JC, Bremmer-Bout M, Janson AA, et al. Antisense-induced exon skipping restores dystrophin expression in DMD patient derived muscle cells. Hum Mol Genet 2001;10:1547–54.

[7] Aartsma-Rus A, Janson AA, Kaman WE, et al. Antisense-induced multiexon skipping for Duchenne muscular dystrophy makes more sense. Am J Hum Genet 2004;74:83–92.

[8] Aartsma-Rus A, Janson AA, van Ommen GJ, van Deutekom JCT. Antisense-induced exon skipping for duplications in Duchenne muscular dystrophy. BMC Med Genet 2007;8:43–51.

[9] Arechavala-Gomeza V, Graham IR, Popplewell LJ, et al. Comparative analysis of antisense oligonucleotide sequences for targeted skipping of exon 51 during pre-mRNA splicing in human muscle. Hum Gene Ther 2007;18:798–810.

[10] Lu QL, Mann CJ, Lou F, et al. Functional amounts of dystrophin produced by skipping the mutated exon in the mdx dystrophic mouse. Nat Med 2003;9:1009–14.

[11] Graham IR, Hill VJ, Manoharan M, Inamati GB, Dickson G. Towards a therapeutic inhibition of dystrophin exon 23 splicing in mdx mouse muscle induced by antisense oligonucleotides (splicomers): target sequence optimisation using oligonucleotide arrays. J Gene Med 2004;6:1149–58.

[12] Bremmer-Bout M, Aartsma-Rus A, de Meijer EJ, et al. Targeted exon skipping in transgenic hDMD mice: a model for direct preclinical screening of human-specific antisense oligonucleotides. Mol Ther 2004;10:232–40.

[13] Jearawiriyapaisarn N, Moulton HM, Buckley B, et al. Sustained dystrophin expression induced by peptide-conjugated morpholino oligomers in the muscles of mdx mice. Mol Ther 2008;16:1624–9.

[14] van Deutekom JC, Janson AA, Ginjaar IB, et al. Local antisense dystrophin restoration with antisense oligonucleotide PRO051. N Eng J Med 2007;357:2677–87.

[15] Kinali M, Arechavala-Gomeza V, Feng L, et al. Restoration of dystrophin expression in Duchenne muscular dystrophy: a single blind, placebo-controlled dose escalation study using morpholino oligomer AVI-4658. Lancet Neurol 2009;8:918–28.

[16] Arora V, Devi GR, Iversen PL. Neutrally charged phosphorodiamidate morpholino antisense oligomers: uptake, efficacy and pharmacokinetics. Curr Pharm Biotechnol 2004;5:431–9.

[17] Heemskerk HA, de Winter CL, de Kimpe SJ, et al. In vivo comparison of 2′-O-methyl-PS and morpholino antisense oligonucleotides for DMD exon skipping. J Gene Med 2009;11:257–66.

[18] Gebski BL, Mann CJ, Fletcher S, Wilton SD. Morpholino antisense oligonucleotide induced dystrophin exon 23 skipping in mdx mouse muscle. Hum Mol Genet 2003;12:1801–11.

[19] Alter J, Lou F, Rabinowitz A, et al. Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology. Nat Med 2006;12:175–7.

[20] Malerba A, Thorogood FC, Dickson G, Graham IR. Dosing regimen has a significant impact on the efficiency of morpholino oligomer-induced exon skipping in mdx mice. Hum Gene Ther 2009;20:955–65.

[21] McClorey G, Fall AM, Moulton HM, et al. Induced dystrophin exon skipping in human muscle explants. Neuromuscul Disord 2006;16:583–90.

[22] McClorey G, Moulton HM, Iversen PL, Fletcher S, Wilton SD. Antisense oligonucleotide-induced exon skipping restores dystrophin expression *in vitro* in a canine model of DMD. Gene Ther 2006;13:1373–81.

[23] Aartsma-Rus A, Fokkema I, Verschuuren J, et al. Theoretic applicability of antisense-mediated exon skipping for Duchenne muscular dystrophy mutations. Hum Mutat 2009;30:293–9.

[24] Popplewell LJ, Trollet C, Dickson G, Graham IR. Design of phosphorodiamidate morpholino oligomers (PMOs) for the induction of exon skipping of the human DMD gene. Mol Ther 2009;17:554–61.

[25] Wilton SD, Fall AM, Harding PL, McClorey G, Coleman C, Fletcher S. Antisense oligonucleotide-induced exon skipping across the human dystrophin gene transcript. Mol Ther 2007;15:1288–96.

[26] McCulley C, Adkin C, Fletcher S, Wilton SD, Morgan J, Wells DJ. Optimising the efficacy of morpholino antisense oligonucleotide induced exon-skipping in primary myoblasts using nucleofection, submitted for publication.

[27] 'tHoen PAC, de Meijer EJ, Boer JM, et al. Generation and characterization of transgenic mice with the full-length human DMD gene. J Biol Chem 2008;283:5899–907.

[28] Aartsma-Rus A, De Winter CL, Janson AAM, et al. Functional analysis of 114 exon-internal AONs for targeted DMD exon skipping: indication for steric hindrance of SR protein binding sites. Oligonucleotides 2005;15:284–97.

[29] Aartsma-Rus A, van Vliet L, Hirschi M, et al. Guidelines for antisense oligonucleotide design and insight into splice-modulating mechanisms. Mol Ther 2008;17:548–53.

[30] Harding PL, Fall AM, Honeyman K, Fletcher S, Wilton SD. The influence of antisense oligonucleotide length on dystrophin exon skipping. Mol Ther 2007;15:157–66.

[31] Aartsma-Rus A, Kaman WE, Bremmer-Bout M, et al. Comparative analysis of antisense oligonucleotide analogs for targeted DMD exon 46 skipping in muscle cells. Gene Ther 2004;11:1391–8.

Neuromuscular Disorders

Volume 20 Number 2 February 2010

Cited in: Current Contents/Life Sciences, Elsevier BIOBASE/Current Awareness in Biological Sciences, Index Medicus, MEDLINE, Neuroscience Citation Index, Reference Update, Research Alert, Science Citation Index, SciSearch

CONTENTS

Editorials

Clinical myology at the crossroads; the gospel truth
V. Dubowitz 95

A new section for *Neuromuscular Disorders*: Patients' Forum
V. Dubowitz 97

Review

Ca^{2+} sensitizers: An emerging class of agents for counterbalancing weakness in skeletal muscle diseases?
J. Ochala 98

Research papers

Comparative analysis of antisense oligonucleotide sequences targeting exon 53 of the human *DMD* gene: Implications for future clinical trials
L.J. Popplewell, C. Adkin, V. Arechavala-Gomeza, A. Aartsma-Rus, C.L. de Winter, S.D. Wilton, J.E. Morgan, F. Muntoni, I.R. Graham, G. Dickson 102

Dexamethasone induces dysferlin in myoblasts and enhances their myogenic differentiation
J.J. Belanto, S.V. Diaz-Perez, C.E. Magyar, M.M. Maxwell, Y. Yilmaz, K. Topp, G. Boso, C.H. Jamieson, N.A. Cacalano, C.A.M. Jamieson 111

Two siblings with limb-girdle muscular dystrophy type 2E responsive to deflazacort
L.C. Wong-Kisiel, N.L. Kuntz 122

Muscle phosphorylase *b* kinase deficiency revisited
A. Echaniz-Laguna, H.O. Akman, M. Mohr, C. Tranchant, V. Talmant-Verbist, M.-O. Rolland, S. Dimauro 125

Rigid spine syndrome revealing late-onset Pompe disease
P. Laforêt, V. Doppler, C. Caillaud, K. Laloui, K.G. Claeys, P. Richard, A. Ferreiro, B. Eymard 128

A novel mitochondrial *MTND5* frameshift mutation causing isolated complex I deficiency, renal failure and myopathy
C.L. Alston, M. Morak, C. Reid, I.P. Hargreaves, S.A.S. Pope, J.M. Land, S.J. Heales, R. Horvath, H. Mundy, R.W. Taylor 131

Myositis in Griscelli syndrome type 2 treated with hematopoietic cell transplantation
A.P. Born, K. Müller, H.V. Marquart, C. Heilmann, L. Schejbel, J. Vissing 136

Incontinetia pigmenti-related myopathy or unsolved "double trouble"?
H.B. Huttner, G. Richter, A. Jünemann, W. Kress, J. Weis, J.M. Schröder, A. Gal, A. Doerfler, B. Udd, R. Schröder 139

Workshop report

Inclusion body myositis. MRC Centre for Neuromuscular Diseases, IBM workshop, London, 13 June 2008
D. Hilton-Jones, A. Miller, M. Parton, J. Holton, C. Sewry, M.G. Hanna 142

Patient's forum

The role of patient advocacy organisations in neuromuscular disease R&D – The case of the Dutch neuromuscular disease association VSN
W. Boon, R. Broekgaarden 148

Living with muscular dystrophy: Personal reflections
M.W. Munn 152

WMS 2010 I
WMS online application form II
WMS News III
ENMC Assessment V
Parent project VI

0960-8966(201002)20:2;1-H